Cytotoxic properties of amyloidogenic L68Q cystatin C

A search for therapeutic agents

Indíana Elín Ingólfsdóttir

Thesis for the degree of Master of Science
University of Iceland
Faculty of Medicine
School of Health Sciences

HÁSKÓLI ÍSLANDS

Eitrunareiginleikar mýlildismyndandi L68Q Cystatin C

- Leit að verndandi þáttum -

Indíana Elín Ingólfsdóttir

Ritgerð til meistaragráðu í Líf- og læknavísindum

Leiðbeinandi: Finnbogi Rútur Þormóðsson

Umsjónarkennari: Elías Ólafsson

Meistaranámsnefnd: Bjarni Ásgeirsson

Læknadeild

Heilbrigðisvísindasvið Háskóla Íslands

Maí 2013

Cytotoxic properties of amyloidogenic L68Q cystatin C

A search for therapeutic agents

Indíana Elín Ingólfsdóttir

Thesis for the degree of Master of Science

Supervisor: Finnbogi Rútur Þormóðsson

Masters committee: Elías Ólafsson and Bjarni Ásgeirsson

Faculty of Medicine

School of Health Sciences

May 2013

Prentun: Prentsmiðja, Samskipti

Reykjavík, Ísland, 2013

Ágrip

Mýlildis sjúkdómar einkennast af villu í umbroti á utanfrumu próteinum sem veldur uppsöfnun þeirra og útfellingu í torleysanlega þræði. Eftir því sem meira safnast upp af þessu torleysanlega efni verður vaxandi starfstruflun í þeim vefjum þar sem mýlildið (amyloid) safnast fyrir. Hér er fjallað um tvo mýlildissjúkdóma; arfgeng heilablæðingu (HCHWA-I) og Alzheimers sjúkdóminn (AD).

Arfgeng heilablæðing, sem erfist ríkjandi og ókynbundið, einskorðast við Ísland. Það er punktstökkbreyting í stöðu 68 í cystatin C próteininu sem veldur því að glútamín amínósýra kemur í staðinn fyrir leusín. Þetta veldur uppsöfnun próteinsins og útfellingum innan veggja heilaslagæða sjúklinga. Þessi uppsöfnun veikir æðaveggina sem veldur blæðingu og heilablóðfalli langt fyrir aldur fram.

Alzheimer´s sjúkdómur er heilahrörnunar sjúkdómur sem veldur vitglöpum fyrir aldur fram. Uppsöfnunin mýlildis úr β-próteini, sem myndast vegna misklofnunar á himnupróteininu amyloid precursor protein (APP), myndar svo kallaðar elliskellur (senile plaques) í heilavefinn og fylgir henni vanstarfsemi í taugum þar sem uppsöfnunin á sér stað. Þessi klofnun próteinsins gefur mislöng peptíðbrot þar sem 40 og 42 β-prótein eru algengust. Elliskellurnar í heilavefnum eru frekar úr 40 amínósýra löngu β peptíði meðan að útfellingar í heilaæðum, sem einnig finnast, eru frekar úr 42 amínósýra löngu β-peptíði.

Hvernig mýlildi veldur eitrunaráhrifum er ekki vitað að fullu ennþá. Í Alzheimer sjúkdómi er ekki samband á milli magns mýlildis í elliskellum eða staðsetningu þeirra og alvarleika heilabilunar sjúklingsins. Mýlildið myndast með fjölliðun og í ljós hefur komið að hvert skref fjölliðunar hafa mismikil eitrunaráhrif. Hugmyndir eru uppi um breytingar í starfsemi kalsíum jónaganga, en einnig er oxunarálag þáttur í Alzheimers sjúkdómi. Líkast til eru eitrunaráhrifin samblanda af mörgum þáttum.

Bæði β próteinið í Alzheimer´s sjúkdómi og stökkbreytta cystatin C próteinið í arfgengri heilablæðingu mynda útfellingar í veggi heilaæðanna. Sléttvöðvafrumur framleiða bæði β próteinið og forvera þess. Bæði 40 og 42 amínósýru lengd af β próteini valda eitrunaráhrifum á sléttvöðvafrumur. Stökkbreytta cystatin C próteinið veldur eituráhrifum á sléttvöðvafrumum sem svipar til β próteins eitrunarinnar, en eitrunin er mun fljótvirkari. Þekkt er að þegar β próteini er bætt við ræktanir af taugafrumum verða þær fyrir eitrunaráhrifum þó ekki sé enn vitað hvernig sú eitrun eigi sér stað. Meðal hugmynda má nefna jafnvægistruflunar á kalsíumflæði frumnanna, oxunarálags eða holumyndunar í frumuhimnunni. Umbreyttar PC12 frumur hafa verið notað til að sýna eitrunaráhrif af β próteininu á taugafrumur, en hugsanleg eituráhrif stökkbreytts cystatin C á umbreyttar PC12 frumur eru ekki þekkt.

Helsta meðferð við Alzheimer´s sjúkdómnum felst í hindrun á niðurbroti acetýlkólins. Þessi meðferð tekur á einkennum sjúkdómsins en ekki orsökinni, sem tengist uppsöfnun á mýlildis efninu. Engin meðferð hefur fundist fyrir sjúklinga með arfgenga heilablæðingu en mikil þróun er á lyfjameðferðir fyrir AD sjúklinga. Því hugnast vel að rannsaka hvort lyf fyrir AD sjúklinga gætu nýst sjúklingum með arfgenga heilablæðingu.

Humanin er stutt peptíð sem greinst hefur í heila Alzheimer´s sjúklings og hefur reynst hafa fjölþætt taugaverndandi áhrif. Þar á meðal gegn eitrandi áhrifum sem β próteinið hefur á PC12 frumur. Einnig hefur verið sýnt að humanin getur bjarga sléttvöðvafrumum frá eitur

áhrifum β próteinsins, en áhrif humanins til að hindra eituráhrif stökkbreytta cystatin C próteinsins á sléttvöðvafrumur og PC12 frumur hefur ekki verið kannað.

Vítamín E er áhrifamikið andoxunarefni og talið geta hægt á gangi Alzheimer´s sjúkdómsins. Það minnkar frumudauða af völdum β próteinsins á taugafrumum í rækt, líkt og PC12 frumur, en hindrar samt ekki myndun mýlildisins. Ef ræktaðar sléttvöðvafrumur voru meðhöndlaðar með vítamíni E þá sýndu þær frumur meiri mótstöðu gegn β prótein eitrun. Áhrif vítamíns E á eitrun af völdum stökkbreytts cystatin C hefur ekki verið rannsökuð.

Tramiprosate er efni þróað til að líkja eftir brennisteins glýkósamínóglýkönum sem geta bundist uppleyst β próteins og þannig hindrað fjölliðun þeirra og mýlildis myndun. Hvernig Tramiprosate verkar er samt ekki að fullu vitað. Áhrif Trampiprosate á frumu eitrun af völdum stökkbreytts cystatin C hafa ekki verið rannsökuð.

Abstract

Amyloid diseases are characterized by a misfolding of extracellular proteins, causing them to aggregate and deposit as insoluble fibrils. These deposits can cause functional problems in the tissue they deposit in. Two such diseases are hereditary cerebral haemorage with amyloidosis – Icelandic type (HCHWA-I) and Alzheimer's disease (AD).

HCHWA-I is an autosomal dominant form of an inheritable amyloid disease that is restricted to Iceland. There is a point mutation at position 68 in the cystatin C protein, resulting in a substitution of glutamine for leucine, and causing aggregation and deposits mainly inside the vessel walls of brain arteries in patients. This accumulation weakens the vessel walls causing hemorrhage that leads to a stroke in affected individuals. The onset of clinical symptoms is usually at an early age with mean life expectance about thirty years.

Alzheimer's disease is a neuropsychiatric disorder that causes a progressive dementia in the elderly. There is an accumulation of both amyloid plaques and neurofibrillary tangles. This causes a dysfunction in the neurons where this build-up is seen. The amyloid deposits have been shown to be made up of an extracellular peptide known as amyloid β peptide. The Aβ comes from cleavage of the transmembrane amyloid precursor protein. A miscleaving in AD patients form amyloid β (Aβ) of different lengths, which are more prone to form insoluble fibrils. The most common forms are Aβ40 and Aβ42. Amyloid deposits in plaques are mostly made of Aβ40 while deposits inside the vessel walls are rather from Aβ42.

The process of cytotoxicity is not fully understood. Regarding accumulation of Aβ peptides in amyloid plaques there is a lack of correlation between the amount and location of the plaques and the severity of cognitive decrements in patients. The amyloid fibrils are formed through an oligomerisation pathway and it is thought that different products along this pathway have different toxicity potency. It has been postulated that this toxicity is caused by changes in calcium channel ion influx. The Aβ peptide has been known to form pores in cell membranes, which can act as functional ion channels. Oxidative stress is one of the characteristics in Alzheimer's disease, which leads to excessive reactive oxygen species production. There is most likely not a single mechanism to explain all the aspect of amyloid cytotoxicity.

Both the Aβ peptide in AD and the variant cystatin C in HCHWA-I is seen deposited within the walls of cerebral blood vessels. Smooth muscle cells have been seen to synthesize APP and form both Aβ40 and Aβ42 peptides that induce toxicity in the smooth muscle cells. The toxic effect of the variant form of cystatin C is similar to that seen with the Aβ peptide on smooth muscle cells, except that the toxicity is observed much earlier with the variant cystatin C form.

When Aβ is added to cultures of neuronal cells there is a clear cytotoxic effect. By which mechanism the Aβ induces this toxic effect is not yet known, but is thought to involve disruption of the intracellular homeostasis of calcium, oxidative stress or channel formation in the cell membrane. The PC12 cells are used as an *in vitro* model to study Aβ peptide neurotoxicity and possible protective agents but have never before been used to study possible neurotoxicity from the cystatin C variant.

Most treatments for AD focus on enhancing the levels of neurotransmitters that are depleted through the progress of the disease. These drugs are mainly inhibitory for the enzyme that breaks down acetylcholine. This treatment reduces the symptoms temporarily, but does not address the problem itself, which is the accumulation of the toxic amyloidogenic fibres. For HCHWA-I, there is no treatment.

Humanin is a recently discovered neuroprotective peptide that was found in the occipital region of an AD brain. It is reported to show a variety of neuroprotective effects, including suppression of neurotoxicity elicited by the Aβ peptide on neuronal cells, such as PC12 cells, through cell surface receptor binding. There is also a report on the ability of humanin in rescuing cerebralvascular smooth muscle cells from Aβ peptide induced toxicity. So far the effect on humanin on variant cystatin C toxicity has not been determined.

Vitamin E is an essential nutrient in humans and a powerful antioxidant. It is thought to slow down the progression of the AD in patients. Vitamin E can reduce Aβ induced cell death in neuronal cells, such as PC12 cells. Vitamin E does not inhibit the Aβ fibril formation. Smooth muscle cells treated with vitamin E has shown to protect them against Aβ assaults. The effect of cystatin C toxicity and antioxidants has not yet been determined.

Tramiprosate is a compound developed to mimic sulphated GAG and interact with the soluble Aβ to hinder the formation of fibrils. The exact mechanism of Tramiprosate action is not yet fully known. The affect of Trampiprosate on cystatin C toxicity has not yet been determined.

Acknowledgements

The work in this project was performed at the Faculty of Medicine and the Science Institute of the University of Iceland. The project was funded by the Icelandic Research Fund (Rannís) and Heilavernd (HCHWA-I research fund).

First of all I want to offer my thanks to my supervisor, Finnbogi R. Thormodsson, for giving me the opportunity to work with him on this project, for teaching me all I know about research work, the pride in good culture work and joy of conclusive result. For all his guidence, instructions and support he showed me during the work. I would also like to thank Bjarni Ásgeirsson for his guidence and instruction for the biochemical work and help on writing this thesis and my thanks go to Elías Ólafsson for his help on this thesis.

Furthermore, I would like to thank the people at the Faculty of Medicine that gave me support in this research. Expecially the following individuals: Anna Thóra Pétursdóttir, for her help with the PC12 cell lines and an undying support, vital encouragement through out this process and good friendship. Helga Eyja Hrafnkelsdóttir, for teaching me cell culture tecniques, endless support and invaluble times in the hood. Guðrún Jónsdóttir for her invaluble help with the PC12 lines and good cheer. Marteinn Thór Snæbjörnsson, for all his help and advise. The people at the Stem Cell Research Unit (SCRU) for their help, advice and unselfish sharing of reagents. Jón M. Einarsson for his good nature and patience when helping with statistical analysis. Jóhann Arnfinnsson for teaching me the teqnuices and value of photo development. Pétur Henry Pétursson, for his instructions and advice. And also all the other people at that supported me in so many ways.

I would like to thank Jorge Ghizo for inviting me to study under him at the NYU Medical School in New York city. For all his guidence, support and wisdom. Agueda A. Rostagno for teacing me immunohistochemical work and the endurable useage of a pressure cooker. Einar Sigurðsson for advicement and providing Aβ peptides for the project. David Bernstein for taking me under his wing and showing me the ropes and Emiliya Mezhericher for all her help.

Last but not least I want to thank my father, Ingólfur Örn Herbertsson. Who has always been a strong supporter of all that I do and because of his kindness I aspire to greater things. Bryndís Brandsdóttir, who has been my greatest role model and shown me what I could aspire to. Björn Vidar Adalbjörnsson for caring support, life savings and invaluble help with writing this thesis.Ingþór Ingólfsson for his mental support and help with artwork. I would also like to thank all my friends that have listened and supported me with patience and encouragement.

Contents

Figures and tables

Figures:

Tables:

Abbreviations

3-APS	3-amino-1-propanesulfonic acid
Aβ	amyloid β-protein
AD	Alzheimer´s disease
ADDL	amyloid β derived diffusible ligands
ALS	amyotrophic lateral sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole
Apo E	apolipoprotein E
Apo J	apolipoprotein J
APP	amyloid precursor protein
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAA	cerebral amyloid angiopathy
CSF	cerebral spinal fluid
CST3	cystatin C gene
ddH_2O	double distilled water, ultra pure water
DMSO	dimethyl sulfoxide
FAD	familial Alzheimer´s disease
FBS	fetal bovine serum
GAG	glycosaminoglycan
HCCAA	hereditary cystatin C amyloid angiopathy
HCHWA-I	hereditary cerebral haemorrhage with amyloidosis – Icelandic type
HN	Humanin
HNE	4-hydroxynonenal
NFT	neurofibrillary tangles
NGF	nerve growth factor
NMDA	N-methyl-D-asparate
PBS	phosphat buffer solution
PHF	paired helical filament
PUFA	polyunsaturated fatty acid
PS1	presenilin-1 gene
PS2	presenilin-2 gene
RAGE	receptorfor advanced glycosylation end-product
ROS	reactive oxigen species
SDS	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMC	smooth muscle cell
vitC	vitamin C
vitE	vitamin E

1. Introduction

1.1. Amyloid diseases

Amyloid disease´s, or amyloidosis, are characterized by specific extracellular proteins, which misfold or fail to remain correctly folded, causing the proteins to aggregate and deposit as insoluble fibrils [1]. There are more than 20 proteins, or their proteolytic products, which are recognized to form amyloid fibrils and be causative agents in diseases. Around half a dozen of them are implicated in diseases of the central nervous system [2]. There are more than 20 degenerative conditions recognized that involve amyloid formation affecting either the nervous system or other organs of the body. The most commonly known are Alzheimer´s disease (AD) and the prion diseases such as Creutzfeldt-Jakob disease [3]. Deposits of the amyloid can be sporadic, familial or transmissible. Sporadic amyloidosis appears randomly in the ageing population. As we age, the homeostasis for protein clearance in cells decrease and amyloid deposits can start to form although there is no predisposing condition except old age [4]. Familial amyloidosis is a hereditary condition, where gene mutation causes misfolding of a protein variant. Transmissible amyloidosis is a form of amyloidosis caused by an infection of a pathogen, which is not always completely characterized. The transmissible amyloidosis is what is found in prion diseases or from atypical viruses [3, 5, 6]. Amyloidosis can also be an acquired condition, for instance in patients who undergo a long term haemodialysis [7]. Amyloidosis is therefore a wide range of diseases that can differ greatly both clinically and in their manifestation.

When a large amount of fibrillar material deposits in a tissue, it can subvert the tissue architecture and consequently cause a dysfunction in the involved organ. Amyloid fibrils can also cause dysfunction to the organ by interacting or interfering with cell receptors [8]. The amyloid hypothesis [9] states that there is a causative link between the formation of these amyloid aggregates and the pathological symptoms [10]. There seems to be a fine balance between the rate of amyloid formation and the amyloid clearance and the amyloid deposits can be re-absorbed and the organ dysfunction reversed if the amyloid progress is shut down. Also if the pathogenic process is halted it might be sufficient to reduce the concentration below the concentration threshold needed to start oligomerisation [1].

By identifying the pathogenesis for each of the amyloid diseases, an insight could be gained that might help in reversing or hindering the process.

1.1.1. Amyloid

Amyloid refers to any amorphous, congophilic, extracellular insoluble protein deposits. These deposits are made of misfolded proteins, which in their natural form are soluble. These deposits show birefringence with polarized light when stained with congo red [2, 11]. With electron microscopy it can be seen that amyloid consists of rigid, nonbranching straight structure of uniform fibrils, 6-10 nm in diameter and with a smooth surface that is made from highly organized β-pleated sheet protein fibrils [12, 13]. Amyloid also shows green fluorescence when stained with thioflavin S. Both the congo red

staining and the thioflavin S staining are considered specific for amyloid, since they are both dependant on the high β-sheet content of the protein [2].

Different amyloid fibrils have no apparent amino acid sequence homology and no similarities in size or structure. It is a general property of the polypeptide chain, which enables the amyloid formation and not a specific interaction of the amino acids side chains. Therefore, any protein under the right circumstances, can form amyloid fibrils [1]. Proteins that are unrelated to any amyloid disease have been made to aggregate *in vitro* to form structures indistinguishable from the amyloid fibrils, which are known to cause diseases [14]. In vitro research has shown that peptides as few as 4-6 residues can form well defined fibrils with the same amyloid properties as a protein with 100 residues or more. This also gives strong support to the notion that the ability to form amyloid fibrils lies in the physio-chemical properties of the polypeptide main chain rather than any interaction between side chains [15].

1.1.2. Amyloid formation

All environmental factors that can interfere with the three-dimensional structure of the protein can increase the risk of amyloid formation. By destabilizing the soluble native proteins structure, several factors can influence the fibril formation process. These factors can be variations in environmental conditions such as low pH or high temperature, lack of specific ligands, moderate concentration of salts, chemical modification like oxidation or proteolysis, and interactions with metal ions and osmolytes [3]. Thankfully, the formation of stable amyloidogenic protein aggregates does not normally occur *in vivo* under physiological conditions. Usually, some change in conditions is needed to trigger the formation of amyloid aggregates.

There can be a few different reasons for this misfolding. Firstly, there may be an intrinsic propensity for this particular pathologic conformation. We can see that in an aging brain, where normal transthyretin deposits as amyloid in senile systemic amyloidosis [4]. The amyloidogenic protein may have to reach a critical concentration to trigger fibril formation. For instance, deposit formation can be because of a high concentration of a healthy protein, for example in patients undergoing long term hemodialysis there is a risk of β_2-microglobulin deposition in the kidneys [7]. Secondly, protein alteration can also initiate amyloids formations. A point mutation in the coding region can result in an amino acid substitution that subsequently can convert the native protein to a fibrillary form. Replacement of a single amino acid occurs in hereditary amyloidosis [16] that consequently results in formation of insoluble protein fibrils. Thirdly, there might be a proteolytic remodeling of a protein precursor that can initiate amyloid protein formation. Best recognized of such proteolytic miscleaving is the β-amyloid precursor protein. These mechanisms can work independently or can supplement each other [17].

These partially unfolded or misfolded proteins are much more prone to aggregation then their native protein forms. An increase in concentration of aggregation prone proteins can occur as consequence of enhanced synthesis or a reduced clearance [3]. Whatever starts the fibrillitation pathway, the end product is an irreversible and mature fibril aggregate that interferes with the tissue integrity.

Number of amyloid associated proteins or chaperons are known to be colocalized with the amyloid deposits without being part of the fibrils themselves. Known amyloid associated proteins are serum amyloid-P component, apolipoprotein E (apo E), apolipoprotein J (apo J), vitronectin, α_1-anti-chymotrypsin, complement proteins, glycosaminoglycan and other extracellular matrix proteins [17].

1.2. Cerebral amyloid angiopathy

Cerebral amyloid angiopathy (CAA) refers to a pathological process where an amyloid protein is progressively deposited in the cerebral blood vessel walls causing degenerative vascular changes [18]. All forms of CAA are associated with stroke and/or neurodegenerative conditions, a major consequence of the latter would be dementia. Stroke is more often found in the form of cerebral hemorrhage and much less frequently as an ischemic infract. Dementia is usually multifactorial, and can in many cases be traced to the presence of amyloid within the vessel walls [19].

CAA can occur as either hereditary or sporadic amyloid diseases. The hereditary form accounts for the minority of CAA cases, but are more diverse. The sporadic form is overwhelmingly associated with advancing age [20]. CAA is a common cause of cerebral haemorrhage in the elderly and non-hypertensive individuals. This is a non-traumatic primary cerebral haemorrhage producing stroke. CAA is rarely seen before the age of 50 but subsequently shows steady increase with advanced age and autopsy data has confirmed that by the age of 90 over 50% of the population is inflicted with some form of sporadic CAA [21].

CAA is a pathological condition with amyloid deposition within the media of small arteries and arterioles, veins and capillaries. The leptomeningeal and cortical vasculature is most affected. There is a characteristic acellular thickening of the vessel walls in small and medium sized arteries and arterioles but the veins are often less affected [22]. The amyloid fibrils replace smooth muscle cells in the media that can cause separation of the internal elastic membrane and the external basement membrane [23].

The formation of amyloid in the blood vessel walls is a multi step process. In sporadic CAA, amyloid initially appears in the basement membrane around the smooth muscle cells in the abluminal area of the media and adventitia, this gradually spreads toward the internal elastic lamina of arteries and the endothelium. This follows a progressive loss of smooth muscle cells as the vascularature degenerates, yet the endothelial cells remain unaffected. In milder forms of CAA, amyloid has been observed in the media without any significant destruction of the smooth muscle cells. However, in moderate CAA the smooth muscle cells are often absent and in severe CAA there is substantial loss of smooth muscle cells in the adventitia, with microaneurysms around the site of dysfunction, fibrinoid necrosis and leakage of blood through the wall [2, 24].

The amyloid accumulation in the cerebral vessels generates neurovascular dysfunction, which leads to loss of vascular function, smooth muscle cell destruction and the breakdown of the vessels integrity. This can produce recurrent and eventually fatal intracerebral haemorrhage. In severe neurovascular dysfunction there might be chronic white matter ischemia before there are significant

changes in the vascular structure. However, vascular rupture and spontaneous haemorrhage appears in advanced stages of the disease. By then there is a significant loss and compromise to the structural integrity of the affected arterioles [25]. Amyloid laden vessel walls will become brittle, structurally compromised and eventually unable to withstand either trauma or changes in blood pressure. Patients with CAA can have CAA related haemorrhage after a minor head injury or after an uncomplicated neurosurgical procedures like shunt replacement [26].

CAA associated cerebral bleeds are most often lobar bleeds within the cerebral hemispheres. They rarely happen in the cerebellum and almost never in basal ganglia, thalamus or the brainstem, but those are common sites of bleeds in hypertensive haemorrhages [19]. Haemorrhage tends to occur at the same age for both men and women. Patients can have both hypertensive and CAA microvascular changes with the high blood pressure accelerating CAA related haemorrhage and vice versa. Despite the well establish link between CAA and brain haemorrhage it is hard to identify the infiltrated micro vessels at the site of bleeding, due to the tissue destruction that occurs as consequence of the stroke [18].

1.3. Cysteine proteases, cystatins and cystatin C

Cysteine proteases are proteolytic enzymes that use a reactive cysteine residue at their catalytic site to cleave peptide bonds. An example of cysteine proteases is papain and cathepsins, such as cathepsins B, H and L, which play a major role in cell protein turnover. The cells use cysteine proteases to modulate various pathological processes, such as inflammation and tumors. For example, they have a role in apoptosis, MCH class II immune response, pro-hormone processing and accelerated collagen and elastin degradation [27].

Cystatins are endogenous inhibitors of papain-like cysteine proteases and they inhibit those cysteine proteinases and protect the host tissue from destructive proteolysis either from the host's cysteine proteinases or from cysteine proteinases of viral or bacterial origin [28, 29]. The cystatin superfaimly is devided up into several families based on their location, size and complexity of polypeptide chains. Cystatin C is a family 2 cystatin. Cystatins in family 2 are mainly found in body fluids but to, some extend, also within organ tissue. They contain two highly conserved disulfide bridges and are synthesized as a precursor with a signal peptide. Also, they are secreted from the cell to inhibit exocytosed cysteine proteases with an inhibition constant ranging from subpicomolar to nanomolar [30].

The cystatin C gene (CST3) sequence has been determined [31]. It consists of a 1.2 megabase segment on chromosome 20p11.2 and the coding sequence has 120 amino acid residues, which makes the secreted peptide about 14 kDa in molecular weight. The gene has two introns and three exons [32, 33]. There are three amino acids at the N-terminus of cystatins, which play a role in binding the target protein to the enzymes binding site. There is a conserved glycine in the third postition which is important to orientate the active site on the targeted cysteine protease [34]. Cystatin C that is truncated by its first 11 amino acids, which will contain this conserved glycine, will have decreased affinity for inhibiting human cathepsins B, H and L and also bovine cathepsin S [35]. A

polymorphnuclear leukocyte elastase cleaves human cystatin C within this N-terminal motif and it is known that by cleaving at glycine$_{-11}$ the inhibition of papain weakens by more than 1000 fold [36].

All cells of the body, except red blood cells since they lack a nucleus, express cystatin C. The highest level of cystatin C expression is found in the seminal vesicles producing high concentration in seminal fluid, but there is also elevated concentration in cerebrospinal fluid, salvia, tears, urine, milk and synovial fluid [32, 37]. Cystatin C has shown itself to be multifunctional, being able to inhibit cysteine proteases of both the host and microbial origin, and also showing evidence of range of other functions. Rat cystatin C can induce proliferation of hippocampus-derived neural progenitor cells and does so with a mechanism non-related to its protease inhibition [38]. Cystatin C seems also to have a role within our immunology system, since it has a protective defense mechanisms by blocking the replication of poliovirus, corona virus, herpes simplex virus and streptococcus group A [28, 29, 39]. There is only one know disease which is known to be caused by a dysfunctional cystatin C, and that is hereditary cerebral hemorrhage with amyloidosis - Icelandic type.

1.4. Hereditary cerebral hemorrhage with amyloidosis – Icelandic type

Hereditary cerebral haemorrhage with amyloidosis – Icelandic type (HCHWA-I), also called Hereditary cystatin C amyloid angiopathy (HCCAA), is an autosomal dominant form of CAA restricted to Iceland. Two hundred and twelve patients from nine families have been identified, all originating from the same geographical area, which is a bay area in northwest of Iceland, called Breiðaförður [40-43]. With genetic testing and growing awareness there has been extreme reduction in births of carriers, with the mutated gene, in resent decades. The carrier gene is extinct from all except three of the families [40].

The clinical symptoms of HCHWA-I are mainly a stroke in a previously healthy adult at an early age in life. The onset of clinical symptoms usually begins when patients are in their twenties or thirties. There can be a sudden appearance of symptoms from intracerebral hemorrhage that can differ depending on the location of the hematoma. Usually, there are no preceding events detected that seem to be the cause. It is common that the patient survives the first haemorrhage, but then goes on to have further strokes. If the patient survives these insults a multi infarct dementia might appear, even with paralysis. Death usually occurs within 10 years from the onset of clinical symptoms. The average lifespan of HCHWA-I patients is around 30 years of age and most patient do not reach the age of forty [40, 41, 43].

1.4.1. A variant form of cystatin C.

The HCHWA-I amyloid is made from a variant form of cystatin C [16, 44]. The cystatin C gene is approximately 4500 bp and is located on chromosome 20. The gene is made up of three exons, interrupted by two introns, 2252 bp and 1254 bp, with the junction at nucleotide triplets encoding amino acids in the positions 55-56 and 93-94 [31, 45, 46]. Genomic DNA library was procured from an HCHWA-I patient and the cystatin C gene sequenced. There is a point mutation resulting in a single base substitution, CAG instead of CTG. This results in a change in the amino acid in 68th position of

the protein, glutamine for leucine (L68Q) [16]. This also results in an abolishment of an *AluI* restriction site in the cystatin C gene that is found in healthy individuals [31, 47].

The cystatin C variant, isolated from the amyloid material of HCHWA-I patients, differs from the native form in more than just this amino acid substitution. The protein also lacks the first ten amino acids from the N-terminal. This causes the protein to consist of 110 amino acids instead of 120 like the native protein [16]. This truncation reduces the molecular weight to 13,260 Da [48, 49] from a 13,343 Da, the size of the native protein. When the cystatin C gene from a HCHWA-I patient is sequenced it is intact, so this truncation is most likely a post-translational modification [31]. Monocytes isolated from HCHWA-Is patients were shown to secrete a full length cystatin C indicating that the truncation happens most likely in the amyloid fibril formation process [49]. When the variant or the native cystatin C gene was transfected into human kidney cells, both the cell lysate and conditioned media contained only the full length cystatin C [50] and when cystatin C is isolated from the cerebral spinal fluid (CSF) from healthy individuals, there is no sign of truncation [51, 52]. However, when the CSF of HCHWA-I patients is examined, there is a minor truncation of 8 amino acids [49] and when urine of healthy individuals is analysed there can be found a truncation of either 8 or 9 amino acids from the N-terminal [53].

Similar truncation, as is found in the amyloid, can be produced with leucocyte elastase *in vitro.* Neutrophile elastase can rapidly hydrolyse the bond between residues Val_{10} and Gly_{11} of the wild type cystatin C *in vitro*. Both cystatin C and the leucocyte elastase are extracellular proteins, further indicating that the truncation is likely a post translational effect [36]. It is still unknown what role, if any, this truncation has on amyloid fibril formation or at which state in the process it takes place. The truncated cystatin C still has inhibitory function, which has been demonstrated against papain, even thought the first 10 amino acids are missing, but there is reduced inhibitation of papain and human cathepsin B, L and H [36, 54]. It is likely that the N-terminal truncation of L68Q cystatin C happens at all stages of aggregation, yet do not contribute to the aggregation itself [55].

There is no indication that the N-terminal truncation is altering the protein stability in a way that could lead to higher forms of aggregation. The truncated form of L68Q cystatin C displays a similar folding as untruncated L68Q cystatin C. Therefore it is unlikely that the N-terminal truncation is playing a major role in the folding process. Both truncated and untruncated forms of variant cystatin C, when exposed to slightly denaturing conditions, have shown to have two intermediates forms that are stable and similar between truncation and non-trunctation [55, 56].

1.4.2. Dimerisation of cystatin C

An *E.coli* bacterial expression system has been set up to express the variant form of cystatin C. The variant form of cystatin C has a much higher propensity to dimerase than the native form. It is thought that those physical changes in the cystatin C protein might cause it to be trapped intracellular and not be secreted from the cells as efficiently as the native cystatin C [55, 57]. Dimeric cystatin C is not as effective as a proteinase inhibitor, causing the extracellular inhibitory activity to decrease, and therefore lead to enhance local proteolytic activity [57]. The dimerisation seems to be a concentration

dependent and the native unaltered cystatin C protein can also form dimers but requiring higher concentration for dimerisation than the variant form of cystatin C. The HCHWA-I variant form needs only 75-100 ng to form dimers whereas the native form needs a concentration of 250-500 ng [50].

Since cystatin C loses its inhibitory potential immediately after dimerisation, it is very likely that the active site is involved. The dimers and aggregates are formed at normal body temperature for the variant form, but needed 25°C higher temperature for the native form to form dimers [57]. The most likely site of dimerisation is in the lyzosomes of the cell that have pH of 4,6-5,0, that corresponds the pH range where recombinant cystatin C have been known to dimerize *in vitro* [50]. Although both the variant form and the native form, dimerise, it is only the variant form which has been shown to form fibrils *in vitro* [58] for example, a stable transfection of NIH/3T3 cells showed that variant form of cystatin C protein was insoluble and accumulated intracellular in the endoplasmic reticulum [59].

When the variant cystatin C protein dimerises, it exposes a hydrophobic region on the protein surface. Most proteins show reduction in their hydrophobicity when they dimerise, but for the variant form of cystatin C it increases, this is also found with amyloid forming prion proteins [56]. Cystatin C consists of five-stranded anti-parallel β-sheets wrapped around an α-helix [60].

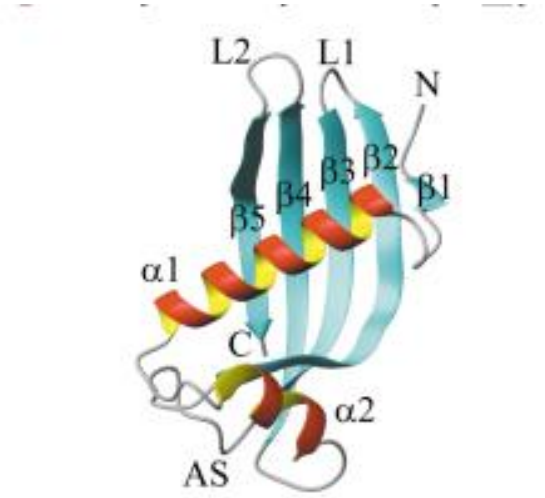

Figure 1.1 3D representation of cystatin C structure. From Janowski R. et al.[61].

Leucine-$_{68}$ interacts very closely with tyrosine-$_{34}$, valine-$_{31}$, alanine-$_{30}$ and leucine-$_{27}$. They are all very hydrophobic amino acids and form a hydrophobic core within the cystatin C protein. The L68Q substitution is therefore in the hydrophobic core of the cystatin C protein and by replacing a hydrophobic leucine with a hydrophilic glutamine, the variant form of cystatin C has a more unfolded structure, causing stretches of hydrophobic residues to be more exposed to the solvent then we find in the native cystatin C. When hydrophobic fragments are exposed to a polar environment, an unfavorable thermodynamic state is formed, leading to oligomerisation and aggregation of the protein. The variant cystatin C form has a more unfolded structure, making it more susceptible to unfolding, proteolysis and fibrillogenesis. Moreover, this partial unfolding exposes also certain residues to further proteolysis, which could lead to further degradation of the protein [58].

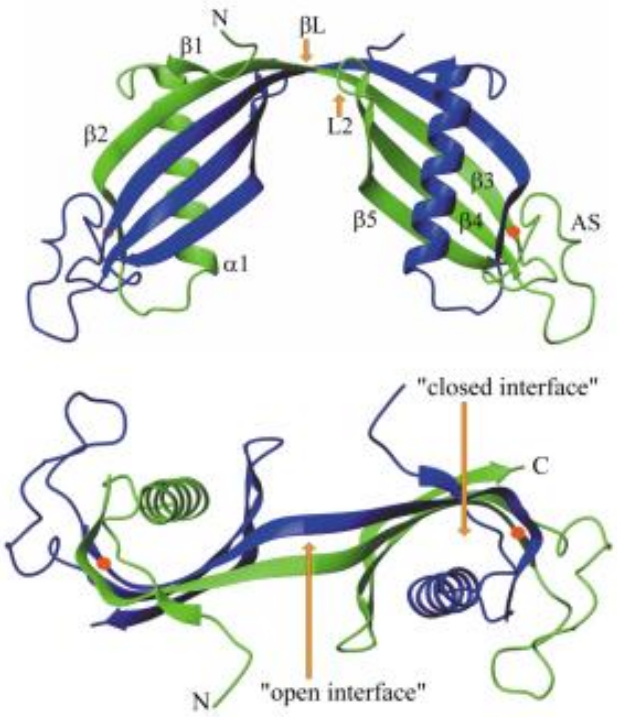

Figure 1.2 Front and top view of a 3D structure of dimerised cystatin C. Domain swapping is shown by separating the cystatin C by color, blue and green. The variant L68Q point mutation is marked in as a red dot. From Janowski R. et al. [61].

The dimerisation is thought to occur through a three-dimensional domain swapping. The L68Q mutation is destabilising the proteins structure, enough to partially unfold the protein and expose the hydrophobic core of the protein to a hydrophilic environment. The partially unfolded monomer seems to be more stable than the correctly folded monomer containing the L68Q mutation. The hydrophobic core is located in a concave groove of the β-sheet and somewhat protected by the α helix (see figure 1.1 and figure 1.2). The longer side chain on Gln, than the Leu side chain, gives a hydrophilic side chain in a hydrophobic environment, which exerts a repulsive force on the α-helix, expelling it form this hydrophobic core. To reduce this unstable formation, two cystatin C proteins can swap the α-strand and one β-sheet with each other (figure 1.2). By this dimerisation, the protein refolds to produce the dimer, a very tight two-fold symmetric dimer, yet retaining the monomeric form of the cystatin C. Domain swapping is a mechanism in oligomerisation and the cystatin C could therefore keep unfolding and refolding to form a higher oligomeric state [61].

1.4.3. Location of the amyloid cystatin C in HCHWA-I patients.

Cystatin C is found in all investigated human extracellular fluids [62]. The HCHWA-I amyloid deposits, are mainly found in small arteries and arterioles of the cerebrum, cerebellum and leptomeninges of the patient's brain [51]. Moreover, tissue specimens from other organs showed anomalous cystatin C immunoreactivity in the medullary sinuses of a submandibular lymph node [51], in the spleen, salivary glands, seminal vesicles and the skin [37]. Adrenal glands showed cystatin C reactivity in their sinusoidal vessels of the zona fasciculata in the adrenal cortex [40]. Intralobular connective tissue in submandibular salivary gland had moderate cystatin C aggregation, yet this aggregation was not seen in the arteries. The central arteries of the splenic corpuscles and its venous sinuses had no indication of cystatin C aggregation. There were no vascular deposits found in other investigated organs such as the dura mater, pituitary gland, submandibular gland, thyroid gland, pancreas, kidney, liver, lung, myocardium, breast, eye, vagus nerve, obturator nerve, external iliac artery or external iliac vein [51].

Post mortem examinations of patient´s brains showed multiple lesions of different ages and sizes representing hemorrhages found in both the cerebral cortex and in the white matter of all the lobes. Organs outside the nervous system have not been shown to have any macroscopic pathologic changes [40, 51, 63, 64]. When observed at a microscopic level, there is a widespread hyalinisation found in the cerebral arteries and arterioles vessel walls. This narrows the vessels and in some cases can cause a complete occlusion. Affected vessels can separate from the media causing aneurysm at those sites. Also, there is a fibrosis in the perivascular tissue. Veins and capillaries are either unaffected or minimally affected [40, 64, 65]. The deposits of cystatin C amyloid in the vascular walls are associated with the loss of smooth muscle cells in the media of the arteries and arterioles [66]. It has been displayed with the loss of α-smooth muscle cell actin immunoreactivity in those vessels. The muscular arteries are more severely affected than the elastic arteries [40].

1.4.4. Cystatin C in cerebral spinal fluid and blood

Cystatin C is found at high concentration in CSF about 5.5 times higher than in blood plasma. In patients with HCHWA-I, the cystatin C concentration was significantly lower or about 2.5 mg/L, which is about a third of what it should be. The concentrations of albumin and IgG unaffected and the same as in healthy individuals [62]. When the proteinase inhibitory capacity was investigated in the CSF of HCHWA-I patients, it was found to be lower than in healthy individuals. The reduced levels of cystatin C could be the cause for this inhibitory decrease. However, the concentration of β_2-microglobulin and kininogen were significantly higher than found in healthy individuals. This might be a regulatory mechanism to maintain the cysteine protease inhibitory capacity of CSF [52]. HCHWA-I patients have a normal cystatin C concentration in their plasma, yet cystatin C dimers could be found when only the monomeric form is found in healthy individuals [67]. The cystatin C isolated from CSF of HCHWA-I patients was not truncated at the amino terminal, like the cystatin C found in the amyloid deposits in the cerebral vessels. This supports the evidence that the truncation is an artifact that happens in the amyloid formation process.

1.5. Alzheimer‘s disease

Alzheimer´s disease (AD) is a neuropsychiatric disorder and a form of amyloid disease, described little more than 100 years ago by Alois Alzheimer [68]. This is a progressive dementing disorder in the elderly with a series of structural abnormalities to the brain. It is also the most common cause of dementia, contributing 60-70% of all dementia cases. It is estimated that there are 30 million people worldwide suffering from this disease, and that number will increase by four times in the next 40 years, making AD one of our major health problems. To date there are no known effective treatments to prevent or delay the onset of AD [69].

Early symptoms of Alzheimer’s disease include confusion, disturbances in short term memory, changes in personality, language difficulties, trouble with maintaining attention and difficulty with spatial orientation. When the disease progresses, the memory loss increases and patients often develop a confusion regarding time and place. In the advance stages of the disease, patients suffer from severe confusion, irritability and even aggression, mood swings and long-term memory loss [68].

Three things make up the hallmarks of AD. There is accumulation in the form of both amyloid plaques and neurofibrillary tangles (NFT), and then there is extensive neuronal loss in the brain [70]. Firstly, a dysfunction appears in neurons in multiple regions of the central nervous system ending in neuronal cell death. This leads to alterations in the synaptic inputs, predominantly in the amygdala, hippocampus and the neocortex. Then, the neurons start to accumulate neurofibrillar tangles in their cell bodies and dendrites, which consist of a paired helical filament (PHF) made from thin filaments with around 10 nm in diameter [71], making a filamentous network. The major component of the PHF is the tau protein [72]. Thirdly, extracellular deposit, composed from 4 kDa peptide, starts to form and accumulating senile plaques. This peptide is the amyloid β protein (Aβ), which forms insoluble fibrils and oligomers. Usually, abnormal neuronal processes can be seen around these senile plaques [73, 74].

In a normal cortex of an elderly individual, there can be found plaques made form amyloid β protein, yet there is an absence of the clinical manifestations, such as dementia, which accompanies AD. By the age of 75 about 25-30% of individuals, with no sign of dementia, which undergo autopsy, are found to have substantial Alzheimer’s lesions-like amyloid plaques, NFT or neuronal loss [75]. The formation of amyloid plaques and neurofibrillary tangles begin therefore long before the clinical symptoms present themselves. The accumulation is thought to start 10-20 years prior to the onset of clinical symptoms in most cases [76]. This indicates that there are additional factors at play that cause the onset of AD than just the formation of the amyloid material.

1.5.1. Amyloid precursor protein

The amyloid precursor protein (APP) is an integral membrane protein that spans the membrane once (see figure 1.3). This is a glycoprotein of the type 1 transmembrane family, which is expressed in several cell types. The N-terminal of the APP projects towards the extracellular space or to the intracellular vesicle space of cell organs such as endoplasmic reticulum, Golgi apparatus or other intracellular endosomes. The C-terminal projects into the cytoplasmic space [77].

The APP gene is a 400 kb long and positioned on the mid portion of the long arm of chromosome 21 [78]. Pre-mRNA of the APP gene can be alternatively spliced, creating several isofoms of the APP, the most common ones being 695, 751 or 770 amino acids [79]. Interestingly, in Down´s syndrome there is a trisomy on chromosome 21, the same chromosome that harbors the APP gene. All individuals with Down´s syndrome show Aβ deposition in the brain by the age of 35 and neuronal degeneration, as well as signs of dementia with the mean onset of symptoms around 50 years of age [80].

The function of APP is not completely clear, yet several functions have been attributed to this protein. APP is highly expressed in the central nervous system by neurons and is thought to have neurothropic and neuroprotective properties [81]. The 751 and 770 amino acid variants of APP are primarily expressed outside the brain and in the plasma. They are thought to play a role in the coagulation pathway [82]. APP is thought to inhibit serine proteases and play a role in cellular adhesion [83]; in axonal pruning and neuronal migration during the development of the nervous system [84]; and even thought to have a function in vesicular trafficking [85]. APP also plays a role in non-neural cell growth, for example in regulating fibroblast growth [86]. There is also a contribution to certain cognitive behaviors, such as learning and memory [87]. But since the APP is highly abundant in the brain and by altering APP biology, such as is the case in AD brains, there are neurofunctional consequences. APP clearly has an important role in neural cell biology.

1.5.2. Amyloid β-protein

Amyloid β is a 4 kDa peptide, which was isolated and identified from amyloid plaques, both derived from AD patients and Down syndrome individuals [74]. Aβ is cleaved from its APP precursor presented on neuronal cells and is found both in CSF and circulating blood. Aβ is relatively abundant in CSF, with a concentration of 10-20 ng/mL and with much lower concentration in plasma [88]. The peptide can be amyloidogenic and accumulates in the cerebral capillaries, arterioles and venules, mainly in the cerebellum, striatum and thalamus areas [89].

It is not known what part if any the Aβ, as a non-aggregated form, plays in the normal human brain. Neuronal activity is thought to regulate the Aβ generation, and in some experimental models, Aβ seems to suppress neuronal activity by interacting with glutamate receptors. Throughout the lifespan of an individual, neurons are producing monomeric Aβ for some unknown, but presumably normal neuronal function [90].

1.5.3. Cleavage of the amyloid precursor protein to form Aβ

In an healthy brain, APP is proteolysed by α-, β- and γ-secretases [77] and this cleavage results in the formation of the Aβ peptide with the Aβ40 being the most abundant Aβ form generated [91]. There are three major cleavage sites within the APP, corresponding to these three secretases (see figure 1.3).

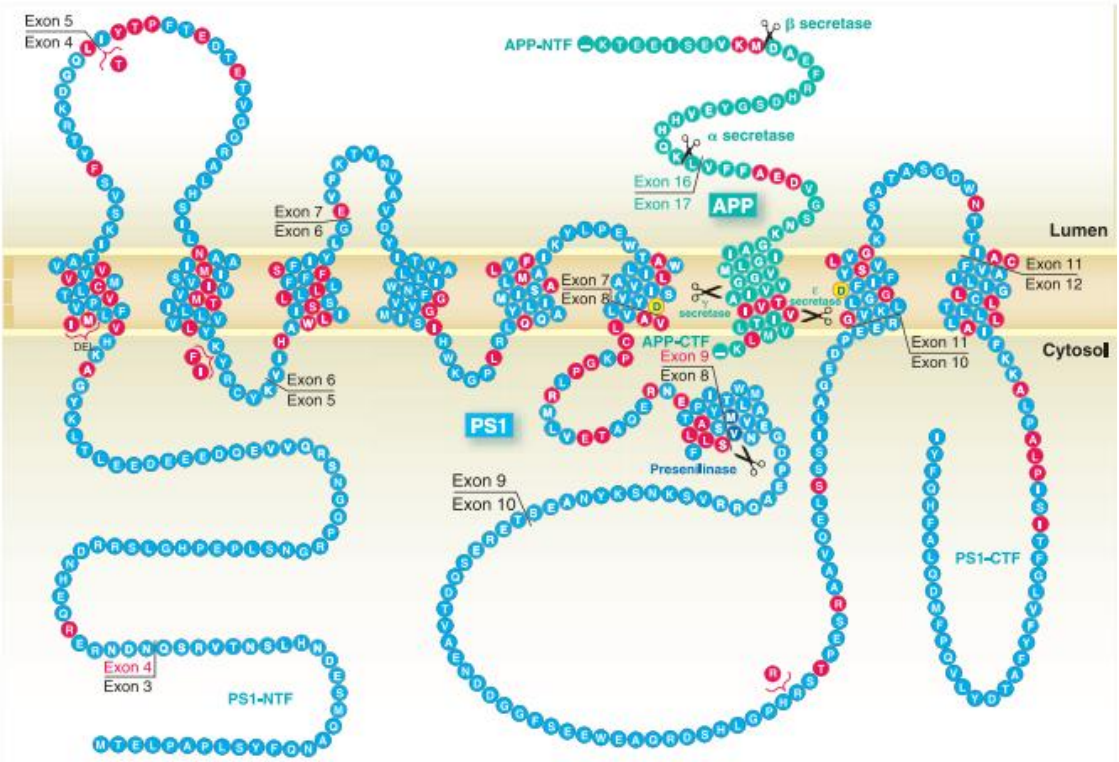

Figure 1.3 The amino acid structure of APP and presenilin-1 and presenilin-2. The APP is shown in green spanning the cellular membrane. The amino acid structure of presenilin-1 and presenilin-2 are shown blue. The cleavage site of α-, β and γ-secretase is shown with scissors on the APP. From Hardy and Selkoe [9].

Proteolysis by the α-secretase does not form the Aβ peptide, but a smaller fragment of the sequence, in the form of Aβ17-40 or Aβ17-42. The main component of the amyloid itself is the Aβ peptide, a 39-43 amino acid long peptide, which is composed of the transmembrane domain portion of the APP and the extracellular domain of the APP. The production of this amyloid forming peptide is made by β-secretase or the γ-secretase proteolysis. γ-secretase cleavage can occur at different sites within the membrane and give a wide range of peptides. Most commonly these fragments are Aβ38, Aβ39, Aβ40, Aβ42 and Aβ43 peptides [88, 92]. Aβ42 is thought to be the main component in senile plaque amyloid, with smaller amounts of Aβ40 mixed in as well. Aβ42 has a higher capacity to self-aggregate than the Aβ40 and can possibly work as a seed to form aggregates with amyloid associated proteins, such as apoE and apoJ [88, 93, 94]. A mutation near the C-terminus in the Aβ region is known to increase the ratio of Aβ42 to Aβ40 but not increasing the total Aβ concentration [95, 96].

1.5.4. Tau protein

Tau is a multifunctional microtubule-associated protein. It aids in the assembly of microtubules and stabilizes the microtubules if needed. Furthermore, tau protein bridges the microtubules with other

cytoskeletal filaments. Tau protein is synthesized and produced in all neurons and in glial cells as well. In a normal healthy brain, there is certain equilibrium between phosphorylation and dephosphorylations of the tau protein. This modulates the stability of the cytoskeleton and also has a role in axonal morphology [72, 97]. In AD patients, there is a hyperphosphorylation of the tau protein. This leads to structural and conformational changes in the tau protein, affecting its role in cytoskeleton organization. Cell bodies of the nerves develop NFT and threads, which are composed of the hyperphosphorylated tau protein [98]. The neuronal dysfunction that occurs in AD is attributed to these neurofibrillary formations. Tau proteins mainly aggregate in the hippocampus, entorhinal cortex and the amygdala [98, 99].

There is a typical progression of the tau pathology in the normal aging brain as well as in the AD brain. It begins in the brain stem and the transentorhinal regions and then progresses through the limbic system and the neocortex [100]. There is a partial overlap of the tau protein and the Aβ protein and there seems to be an interaction between these two proteins [101]. The pathological state of these proteins seem to be able to spread in a prion-like manner within the brain [102]. When isolated brain lysate from either AD patient or AD transgenicly mice, containing human Aβ, is injected into the brain of a mouse that has been transgenicly modified to develop Aβ, then the onset of Aβ pathology starts much sooner than otherwise would happen [103]. Moreover, if a tau protein is injected into mice which would otherwise not form tau pathology, they will start to form tau protein aggregation at the site of injection, which then spreads to neighboring neurons [102].

1.5.5. Familial Alzheimer's disease

Around 5-10% of AD patients have an autosomal dominant inherited mutation causing early-onset of clinical symptoms. This is called familial Alzheimer's disease (FAD), and in these families the onset of dementia is between the ages of 30-60 years. For these patients there is an increase in the accumulation of the Aβ protein that is caused by a mutation in either the APP gene, the presenilins-1 gene (PS1) or the presenilins-2 gene (PS2) [104, 105]. Presenilin-1 and presenilin-2 are transmembrane proteins, which are involved in normal APP processing (see figure 1.3). A mutation will cause problems in cleavage of APP, resulting in a higher ratio of longer Aβ peptides from endoproteolysis of the APP, that are more prone to oligomerisation and fibril formation. This miscleavage by the β- or γ-secretases of the APP with longer Aβ peptides allows amyloid deposits to form in patients' blood vessels of the brain and in that way furthering the progression of cerebral hemorrhages [95].

Mutations in APP are rather rare and in most cases it is a mutation in the presenilin proteins that is the cause for FAD. There are nine known mutation in the APP gene, whereas 75 mutations in the presenilin-1 have been found and three in presenilin-2, all of which cause early-onset AD in affected families. One particular mutation in presenilin-1 causes the most aggressive form of AD with the earliest onset appearing before the age of 50. These mutations in the genes for APP, PS1 and PS2 all cause increased production of Aβ42, rather than its Aβ40 counterpart [106].

1.6. Toxicity and oligomerization of the Aβ peptide

The accumulation of β amyloid protein is found in the amyloid plaques of the AD brain. However, there is a lack of correlation between the amount of deposited insoluble amyloid β protein in these plaques and the degree and location of the neurodegeneration and the cognitive impairment. Indeed, the number of Aβ plaques is not correlated to the severity of cognitive decrements in patients, and there are obvious alterations in the Aβ metabolism and formation of amyloid plaques many years before the clinical symptoms are observed [76]. Furthermore, in the transgenic mice studies, where the mouse model over expresses the APP and/or presenilins, there is still no correlation between amyloid plaques and cognitive alteration or the AD type neurodegenerative changes [107]. The process of the plaque formation is through oligomerization of soluble Aβ monomers. The process is ill defined, but widely accepted as the process for the amyloid formation in amyloid protein diseases. Therefore, the most recent hypothesis state that the soluble Aβ oligomers are mediating the toxicity, which can explain the lack of correlation between the amount of amyloid fibrils and the toxicity [108].

Aβ peptide is released into the body fluids after cleavage. There it remains in a non-toxic form, and does not aggregate. It is made continuously and normally by most cells in the human body throughout life although the exact role of Aβ protein in AD has yet to be clarified [109]. It could be triggering a subsequent neuronal death process or there could be a toxic threshold that needs to be reached before neuronal cell death occurs. Moreover, Aβ could be working as a continual driver of neuronal death. More than 20 years ago it was demonstrated *in vitro,* that monomeric non-toxic Aβ protein could be converted to a toxic species by just incubating it for a few days in a buffered solution [110]. A formation of a high-molecular weight species was seen in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) that has been associated with the toxicity. There is a formation of a Aβ protofibril which has a smaller diameter than the amyloid fibrils with high β-sheet content and are likely a species in the amyloid fibril formation pathway [111]. Aβ protein extraction from AD patient´s brain tissue gives soluble species, which are more strongly correlated with the disease symptoms rather than the amyloid plaque formation [112, 113]. These observations support the hypothesis that soluble Aβ species are involved in the pathogenesis of AD rather than the fully formed amyloid fibril themselves.

The oligomerisation process is complex and not fully understood to date. Between the soluble monomeric form of the Aβ peptide and the fully formed insoluble Aβ fibrils there are many forms of intermediate Aβ oligomers, such as protofibrils, annular structures, paranuclei, Aβ derived diffusible ligands (ADDL) and globulomers to name some. In general, a soluble oligomer is defined as Aβ assemblies that are not pelleted from physiological fluids by high speed centrifugation [114]. Furthermore, soluble oligomers can also bind to other macromolecules or even to a cell membranes and can therefore become soluble [115]. Figure 1.4 shows the schematic view of the oligomerisation process as it is thought to be.

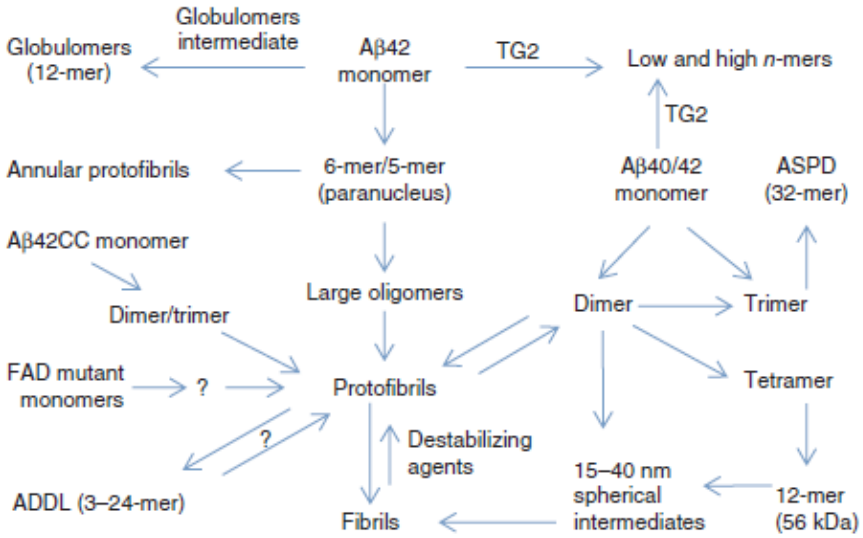

Figure 1.4 A recent schematic view of different natural and synthetic Aβ assemblies according to Benilova, I. et al [108].

A complex equilibrium between monomeric Aβ, oligomeric Aβ and the amyloid fibrils is observed. Several oligomeric forms coexist that can propagate into fibrils, or not. The debate whether there is a distinct toxic species can be misleading [108]. This implies that there is a precise species of oligomers working through specific biological mechanism causing the cellular toxicity. There is an alternative interpretation where there is no one toxic oligomer, but rather a mixture of various oligomers and aggregates, an Aβ peptide „soup" so to speak, which is interacting with each other and causing an interaction with cellular membranes and proteins in a rather non-specific way, causing the toxic cellular effect. The toxicity is related to the heterogeneous character of natural aggregates or to unknown post-translational modification [108].

It is now generally accepted that the Aβ toxicity is neither stemming from the monomer form nor from insoluble fibrils. Rather, the most toxic form comes from soluble structures ranging from small oliogmers to larger assemblies, most often called protofibrils. Protofibrils are intermediates that are flexible and can continue to polymerize *in vitro* and form fibrils, but they can also depolymerise into lower order of species. The protofibrils are just 5 nm in diameter, only half the width of fully formed amyloid fibrils [116]. It has been found that Aβ in a tetramer form is more toxic than the dimeric form of Aβ [117].

Furthermore, recent evidence suggests that the amount of soluble oliogmers of Aβ peptide in the brain correlates better with the severity of the disease rather than the occurrence of the amyloid plaques containing the Aβ fibrils [118]. The soluble oligomers are better correlated to learning and memory deficient in animal models of AD as well [119].

1.6.1. Direct binding of Aβ to a receptor causing cellular toxicity

The identity of a membrane protein receptor for Aβ has been sought for many years [120]. Oligomers are thought to be stable structures that interact specifically with receptors. Soluble Aβ oligomers are thought to interfere with signaling pathways downstream of certain synaptic plasma membrane receptor such as NMDA (N-methyl-D-aspartate) receptors, affecting calcium influx and postsynaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) receptors [121]. Several other cell surface receptors have been considered as binding site for Aβ. These include the tachykinin receptor, serpin-enzyme complex receptors, integrins, P75 neurotrophin receptor and receptor for advanced glycosylation end-product (RAGE) [122-124]. Even though there are receptors that Aβ peptide is known to have some interaction with, the impact of these interactions is not yet known, or their involvement with the toxic potential of Aβ.

It has been postulated that the interaction of Aβ with cell receptors can cause an influx of calcium. Moreover, it is additionally thought that Aβ can directly interact with voltage or ligand gated calcium channels on the cell membrane surface, and also that the toxic effect is caused by an excitatory amino acid on the peptide [125, 126]. However, there are result that contradict this, for example, when calcium channels have been blocked on the cell surface with effective and specific calcium channel blockers, the cells still continue to die when exposed to Aβ peptide [127]. This indicates that the mechanism of this cytotoxicity is too intricate to allowing just blocking of calcium channels to rescue the cells.

1.6.2. Aβ pore or channel formation in cell membranes.

Aβ peptides can adhere to cell membranes and form pores that are able to act as functional ion channels. This hypothesis was originally set forth when the Aβ peptide was found to create ionic current fluxes after Aβ had been incorporated into artificial membranes [128], and is now known as the β-amyloid calcium channel hypothesis. The Aβ peptide can be inserted into the membrane in a structural configuration that forms these channels; most likely with a chain of processes, until the pore becomes large enough, to induce cytotoxicity and cause cell death by apoptosis [129, 130]. The cause is apparently a change in neuronal and endothelial cell membrane permeability [131, 132]. Upon formation of this Aβ pore, there is a loss a calcium homeostasis and an increased influx of calcium into the cell [133].

There is thought to be a minimal polymeric structure, which could directly interact with neuronal plasma membrane and be the cause of neuron loss in AD. Most likely the pore size is made of hexamers [134, 135].

Uncontrolled Aβ pore formation will lead to an irreversible degeneration activating cellular processes that lead to cell death by apoptosis, but not if the formation is blocked [130, 136]. It has even been shown that highly neurotoxic Aβ 25-35fragments, and also full length oligomers, are capable of opening transitional pores in the mitochondria activating the mitochondrial death pathway [137].

1.6.3. Oxidative stress and Aβ

Oxidative stress is implicated in number of disease states, but most notably in those that are correlated with advanced age, and has been termed the free radical theory of aging [138]. Oxidative stress is one of the characteristics of AD. It is defined as the imbalance between biochemical processes that generate to reactive oxygen species (ROS) and the factors responsible for destroying these ROS. This is called the antioxidant cascade. ROS damages all cellular biomacromolecules, like lipids, proteins, sugars and polynucleotides. ROS can also cause secondary products when oxidized that can also cause damage. In extensive oxidative stress, there is a reduction in the levels of antioxidants. The most common antioxidants are the vitamin E (vitE), vitamin C (vitC), along with the proteins glutathione, superoxide dismutase, catalase and thioredoxin. Oxidative stress causes an increase in the oxidative damage to DNA and an increase in lipid peroxidation and protein oxidation [139].

The Aβ peptide causes ROS production in combination with metal ions and oxygen, especially with hydrogen peroxide, which leads to increased oxidative stress and inflammation. The toxic effects are most severe with a soluble aggregated Aβ form. The metal combination gives a moderate oxidant that can generate H_2O_2 when oxidised with O_2 and a reducing agent, for example ascorbate (vitamin C). The aggregated peptide, most likely with bound redox metal ions, is initiating free radical production, which causes lipid peroxidation and protein oxidation, reactive oxygen species (ROS) formation and cellular dysfunction. All of this leads to calcium ion influx and accumulation that subsequently ends in cell death [140].

The nervous system is more vulnerable to the oxidative stress than other tissues. It has a high rate of O_2 utilization, yet it has a poor concentration of antioxidants and related enzymes that protects it from the oxidative stress. There are also high concentrations of redox active transition metals that could generate ROS. When neurons get damaged, we can often see both an increase in oxidative stress, but also a decrease in the antioxidant defenses [139]. It does not help that neuron membranes are particularly enriched in polyunsaturated fatty acids (PUFA) which makes them particularly susceptible for lipid peroxidation [141].

The precise radical species that initiates lipid peroxidation is still uncertain but lipid peroxidation is the beginning of a cascade, which ends in cell death. The lipid peroxidation produces 4-hydroxynonenal (HNE) amongst other reactive carbonyl compounds. HNE is a toxic product of lipid peroxidation and in itself a signal for apoptosis. This secondary toxic product is thought to be less reactive than the radicals, yet half lives range from minutes to hours and they can therefore diffuse from their site of origin and cause damage at distant sites [142].

The aggregated soluble amyloid β-peptide causes the oxidative stress reaction but not the fibrils themselves [143]. There is a consistency with this finding in affected areas of the Alzheimer´s brain and an abnormally high level of redox active metals, such as zinc (Zn), copper (Cu) but particularly iron. An excess of redox active metals is thought at least partially to be responsible for the oxidative damage seen [144]. It is known that both Aβ40 and Aβ42 can cause free radical oxidative stress. A complex made by the Aβ and Cu(II) is known to increase the reduction potential [145].

The accepted model described by Varadarajan, S. et al. [143] is that Aβ peptide, most likely a small soluble oligomer, is inserted into the neuronal and glial membrane bilayer and generates oxygen and redox metal ion dependent free radicals. These free radicals cause the lipid peroxidation and protein oxidations. The membrane damage, which can be because of the Aβ associated free radicals, or just an indirect action of the lipid free radicals, or by the lipid peroxidation product like HNE, leads to cellular dysfunction. The dysfunction can be uncontrolled calcium influx, loss of protein transporter function, disruption of signaling pathways or activation of nuclear transcription factors and apoptotic pathways, all of which end in cell death. As can be seen, there is most likely not a single mechanism that can explain all aspects of oligomer Aβ toxicity.

1.7. Ratio between Aβ42 and Aβ40 and its effect on cellular toxicity

Aβ peptides of different lengths seem to contribute differently to the process of Alzheimer's disease. It is not fully understood why that is at this point in time. It has been shown that alteration in the Aβ42:Aβ40 ratio, in an Aβ peptide mixture, can drastically change its properties, regarding fibrillation and cytotoxity [146].

The most common Aβ species found in serum, CSF and cell culture supernatants is Aβ40 [147]. The Aβ42 is over expressed in FAD and is therefore found in greater concentration in those patients, although still at 10 times lower concentration then Aβ40 [148]. It is thought that the Aβ42 has faster aggregation kinetics, which can be inhibited by the Aβ40 in a concentration dependent manner. This indicate that increasing the ratio of Aβ40 *in vivo* can possibly have a protective effect [149].

It seems that the ratio of the Aβ peptides is more crucial, when it comes to cytotoxicity, rather than the total amount of Aβ peptides [150, 151]. Biophysical parameters *in vivo* can therefore influence the distribution of Aβ toxic and non-toxic species over time [146]. A minor increase in the Aβ42:Aβ40 ratio can create intermediate conformations, which stabilize the toxic oligomeric species [152].

1.8. Interaction between cystatin C and Aβ peptide

Occasionally cystatin C is found co-localized with Aβ in amyloid-laden vascular walls and in the senile plaques in the Alzheimer brain. This co-localisation can also be found in Down´s syndrome, intracranial hemorrhage and cerebral infarction. Furthermore, this kind of co-localization has also been found in the brains of healthy aged individuals [153-155]. When blood vessels from patients with HCHWA-I are immunostained for both Aβ and cystatin C, there is only cystatin C accumulating detecting in those vessels [156]. AD patients showing co-localization of cystatin C and Aβ immunoreactivity in the vessel walls are more likely to suffer brain hemorrhages and have greater accumulation of Aβ amyloid [153].

When amyloid is isolated from leptomeningeal vessels the Aβ peptide is fibrillar while the co-localized cystatin C is soluble and non-amyloidogenic, indicates that the cystatin C deposition is probably a secondary process to the Aβ deposition, yet is increasing the incidents to cerebral

hemorrhages [157]. The Aβ-cystatin C complex isolated for brain homogenates of healthy individuals was found to be SDS resistant and stable, whereas such complex was not detected in homogenates isolated from AD patients [158].

Recent research shows that cystatin C can play a protective role in Alzheimer´s disease by binding to the Aβ peptide and inhibiting oligomerization, fibril formation and deposition of the Aβ *in vitro*. Binding between cystatin C and the soluble, non-pathological form of the Aβ peptide has been found in transgenic mice depositing Aβ peptide, apparently inhibiting plaque formation [158]. Complexes of cystatin C and Aβ have been found in plasma and in CSF of both AD patients and normal individuals. AD patients in general are found to have reduced levels of cystatin C in the CSF [159]. Cystatin C seems to affect the aggregation process of Aβ, but not the total Aβ peptide concentration [158]. Moreover, cystatin C seems to protect neuronal cells from Aβ toxicity directly. If human cystatin C is added to cultured primary hippocampal neurons or neuronal cell lines, along with either oligomeric or fibrillary form of Aβ, the cell survival is increased [160].

The apparent protection from cystatin C is thought to be fourfold. Firstly, cystatin C protects by inhibiting cysteine proteases, which could otherwise cause damage to cells if uncheck, as discussed earlier. Secondly, cystatin C can protect by inducing autopaghy as it has been shown to increase autophagy in cultured cells suffering from nutirtional deprivation or oxidative stress [161] although this has yet been confirmed *in vivo*. Thirdly, cystatin C has been shown to inhibit Aβ oligomerisation and amyloid fibril formation. Specific binding of high affinity is found between the cystatin C and Aβ40 or Aβ42 and cystatin C added exogenously has been shown to protect neuronal cells from cytotoxicity in a concentration dependent manner [159]. Fourthly, cystatin C has been shown to increase neurogenesis. For example, it has been shown when acute hippocampal injury is inflicted on mice that cystatin C expression is increased in the injured area [162]. Also, by adding human cystatin C onto a culture of primary brain cells, there was an increase in glial fibrillary acidic protein, which indicated that cystatin C has a role in regulating glial development [163].

1.9. Cellular toxicity caused by Aβ peptide on smooth muscle cells

Both the Aβ peptide in AD and the mutant cystatin C protein in HCHWA-I are seen deposited within the walls of cerebral blood vessels, mainly in the *tunica media*. Aβ39, Aβ40 and Aβ42 have all been found to be cerebrovascular amyloid [164]. The smooth muscle cells (SMC) have been suggested as a source for both the APP and Aβ peptide in the cerebrovasculature, and degeneration of the smooth muscle cells in the cerebral blood vessel walls accompanies the Aβ peptide deposition [165]. Cultured cerebrovascular smooth muscle cells have been shown to both synthesize the APP and also produce the soluble Aβ peptide [166].

There are evidences indicating vasoreactivity of the soluble Aβ peptide that possibly contributes to the decrease cerebral blood flow observed in AD [167]. Both Aβ40 and Aβ42 have been shown to constrict isolate rat penetrating arterioles, while a reverse peptide Aβ40-1 did not. They appear to directly affect the vasomotor regulation of the arterioles, and ROS production seem to partially mediate these effects [168].

Amyloid deposits are mostly made of Aβ40 rather than the Aβ42 peptide, yet the Aβ42 is thought to be important for inducing the amyloid deposition [164, 169]. When the effects of Aβ40 and Aβ42 on mouse cerebral circulation were compared it was found that only Aβ40 was causing the observed vascular dysfunction [170]. Same was seen in rat aorta, the Aβ42 was less effective [171]. This effect on enhanced constriction by the Aβ40 is thought to be attributed by decreasing the endothelial function but not attributed to effects on the SMC [172]. This is an endothelin-dependent decrease in cerebral blood flow. On the other hand, ATP-induced vasoconstriction seems to be enhanced because of the Aβ42, rather than the Aβ40. Aβ42 is more likely to have a direct effect on the SMC, by enhancing the sensitivity of the SMCs contraction mechanism [168]. When freshly dissolved Aβ40 or Aβ42 was added to endothelial and SMC in culture there was a dose-dependently increased in ROS production in both cell types [173].

When cultured human leptomeningeal smooth muscle cells are incubated with Aβ42 there is an extensive cellular degeneration and in increase in amount of cellular APP and extracellular Aβ levels. This reaction did not happen when incubated with Aβ40 [173]. It seems that it is the soluble form of the amyloid that is toxic form for the SMCs, since when Aβ42 preassembled into fibrils was added to the cell culture, no toxic effect was seen. How the Aβ initiates the pathological cascade of cell death, is still unclear [173].

1.10. Cellular toxicity by variant cystatin C on smooth muscle cells

Amyloid fibrils made from the variant cystatin C (L68Q) accumulates in the *tunica media* of the cerebral blood vessels and as this amyloid material saturates the arterial walls, the SMC disintegrate and disappear, while the endothelial layer appear to remain unaffected [42]. When the variant form of cystatin C was added to a culture of vascular smooth muscle cells a toxic response was seen, showing morphological changes compatible with apoptosis. This toxic effect was shown to be concentration dependent [174] and similar to the one that Aβ is found to inflict on SMC [175]. However, the toxic effect is observed much earlier in the case of the variant cystatin C.

1.11. Cellular toxicity by Aβ or variant cystatin C on PC12 cells

When Aβ is added to cultures of cortical or hippocampal embryonic neuronal primary cells from either rat or human, there is a clear cytotoxicity effect [164, 169]. The neurotoxicity that we have previously seen *in vitro* appears to be because of the insoluble, aggregated form of Aβ [171].

PC12 were originally derived from a pheochormocytoma of the rat adrenal medulla and can be differentiated into a well characterized neuronal-like cells, which is widely used as a model system to study neurotoxicity and neuroprotection [176]. When Aβ is added to cultures of neurons, it can induce both neuronal cell death and render neurons vulnerable to cytotoxicity and oxidative results [177]. By which mechanism the Aβ induces this toxic effect is not yet clear, but it is thought to involve intracellular homeostasis of Ca ions, oxidative stress or channel formation in the cell membrane, as discussed in section 1.6. This model has been used to screen for novel protective agents against Aβ

neurotoxicity. For example, when PC12 cells were treated with Aβ42 for 24 h, a concentration dependent decrease in the cellular redox activity was observed [178] and protection from Aβ neurotoxicity on PC12 cells, by inhibiting oxidative stress, was seen by applying with anamides from *Withania somnifera* fruit [179] or purple sweet potato on Aβ mediated PC12 cell death by inhibiting oxidative stress [179].

The PC12 cells are widely used as an *in vitro* model to study neurotoxicity and to look for possible protective agents against the Aβ toxicity, but they have not previously been used to study possible neurotoxicity of the cystatin C variant.

1.12. Therapeutical approaches for amyloid disease

It is important to know the cause of toxicity in amyloid diseases, like AD and HCHWA-I, in order to know what therapeutic tactic is appropriate when it comes to drug design. Many drugs, which are being developed today, are interfering with the oligomerization pathway. This can be problematic, since it is still unclear what oliogomers are the toxic ones, or if there exists a ratio threshold which causes the toxic affect [108].

One of the main therapeutic targets, for drug design, is the inhibition of the Aβ production itself by identifying small compounds, which can cross the blood-brain barrier, and is able to decrease the production without affecting the β-secretase or γ-secretase activity. This would be a suitable therapy for patients early in the disease, when there has not been a great accumulation of the amyloid and still no sign of dementia. Inhibitors of γ-secretase have been made that have been shown to decrease the Aβ production by 30-40% [180].

Another target is the aggregation pathway, where a small molecule would bind the Aβ soluble monomer and thereby preventing it from forming insoluble oligomers that aggregate. Targeting the oligomerisation pathway can have different consequences depending on at which stage the pathway is halted. This approach could cause accumulation of metastable intermediates of toxic oligomers that would aggravate the disease [108]. Given that this would not happen, the anti-aggregation approach is only targeting pathological events of the disease, and not interfering with normal function such as when the secretases are inhibited.

Another therapeutic approach is using antioxidants, free radical scavengers or calcium channel blockers to work against the oxidative stress [181]. Neurorestorative factors like neurotrophins, to rescue synapses, have also been proposed [182].

Vaccination with Aβ as therapy has been discussed. When synthetic human Aβ peptide was injected into APP transgenic mice, an Aβ antibody was found to cross the blood brain barrier and enter the brain parenchyma [183]. How this was achieved is not clear, but the response to the anti-Aβ lead to enhanced clearing of the Aβ in these mice, even those that had begun to develop senile plaques and symptoms of the disease.

In the future, a combination of these approaches might be used, rather than aiming at just one target. But today, the treatment for AD is mainly to replenish the levels of neurotransmitters that are

depleted by the disease usually by inhibiting the enzymes that break down acetylcholine [184]. Although this treatment might reduce the symptoms momentarily, it does not address the problem itself, which is the accumulation of toxic oligomers. For HCHWA-I, there is no known treatment.

1.12.1. Humanin

Some findings show that there are pro- or anti-aggregation factors in different brain regions that can affect Aβ42 oligomerisation. When healthy neurons are found in an area which is surrounded by oligomers and fibrils, it suggests that they have an intrinsic ability to resist the cytotoxicity of the Aβ. Humanin (HN) is a recently discovered 24 amino acid neuroprotective peptide, which was found in the occipital region of an AD brain through cDNA screening [185]. The occipital lobe, which is rarely affected in AD, shows immunoreactivity when stained for the HN peptide, whereas other regions of the brain do not except for immunoreactive glia cells in the hippocampus of AD brain [186].

HN has been reported to show a variety of neuroprotective effects. It is thought to suppress neurotoxicity of the Aβ42 peptide *in vitro* on both primary neurons and on neuronal cell lines through cell surface receptor binding [185].

HN has been transfected into neuronal cells that also contains genes coding for different variants of amyloid precursors that are associated with FAD. The transfection gave protective effect that increased viability of those cells [187]. Moreover, addition of HN to neuronal cells in culture, rescued the cells from the cytotoxicity of Aβ42. Furthermore, HN has been shown to protect neurons from apoptosis caused by other non-Aβ assaults, such as serum deprivation [188]. When HN was added to non-human cells, such as PC12, there were also protective properties against Aβ toxicity seen [188]. Only HN that is secreted can show neuroprotective effect, not HN expressed and remaining intracellular [185] strengthens the assumption that HN works by binding to a cell membrane receptor.

When AD mice got a direct injection of a large amount of Aβ peptide into their hippocampal area, it caused excessive neuronal death. When these mice also received humanin, in their injections, protection of the neurons was observed [189], along with better short-term memory and performance better in maze studies [190].

HN has also been reported to show protective effects in other diseases as well. In atherosclerosis, the endothelial cells are under toxic attacks from oxidized low-density lipoprotein. These endothelial cells have been found to express HN both in arteries and veins. That seems to protect them from this oxidative stress [191]. Moreover, it has even been reported that humanin can inhibit the neuronal death that is induced by brain ischemia by up regulating the PI3 kinase/AKT signaling [192, 193]. Furthermore, HN was shown to exhibit neuroprotective activity in familial amylotrophic lateral sclerosis (ALS), against the toxicity caused by ALS-related mutant superoxide dismutase [194]. All of this indicates that HN is part of natural defense mechanism in the body against insults, mainly from inflammation and oxidation stress. Interestingly, HN has not been known to show any protective ability against toxic fragments of prion proteins [187].

It has been reported that humanin can rescue human cerebrovascular smooth muscle cells from Aβ induced toxicity [195]. Soluble Aβ induced several pathological responses in human cerebrovascular SMC in culture including fibril assembly at the cell surface and increased expression of APP [196, 197]. This was followed by dramatic morphological changes and apoptotic cell death. If the cells were treated simultaneously with both HN and Aβ, then cell viability testing showed over 95% rescue of cells compared to cultures receiving Aβ treatment alone. Yet, even if the cells were saved from cell death, the Aβ still bound to the cells and formed fibrils with morphological signs of degeneration, indicating that most likely the HN protection is occurring downstream from these cell surface events [198]. This indicates that the HN peptide could also potentially rescue SMC *in vitro*.

The effect of HN on variant (L68Q) cystatin C toxicity has not so far been determined.

1.12.2. Vitamin E (α-tocopherol)

Oxidative stress occurs when there is an imbalance between production of ROS and the clearance of ROS. This imbalance can result from a disease such as AD, but it also occurs naturally with increasing age. To keep the damage of the ROS in check there is an array of antioxidant systems [138, 139].

Vitamin E is an essential nutrient in humans and a powerful antioxidant. It works as a natural antioxidant and scavenges the free radicals in the cell membranes, and protects unsaturated fatty acids form lipid peroxidation. The normal plasma concentration of vitamin E in humans ranges from 11.6 to 30.8 mmol/L [199]. In animal models, it has been found that dietary supplements of vitamin E can increase its concentration in brain areas, for example, when rats got food supplemented with vitamin E it could increase the concentration in their hippocampus by 50-70% [200]. In clinical studies, where vitamin E was given as a dietary supplement, there was a significant benefit when it came to functional assessments, but when it came to cognitive tests, there was no significant benefit of supplemented vitamin E. This correlates with the finding that if vitamin E supplementation is begun later when the amyloid plaques have already formed in AD transgenic mice, there is a reduction in oxidative stress but not change in cognitive symptoms. The vitamin E is thought to slow down the progression of the AD in patients with moderately to severe AD, but works is best when applied in the early in the stages of the disease [201].

There are reports that indicate that there is an increased vitamin E concentration in AD patient´s brain. Indicating a compensation response against increased ROS production [202]. However, other researchers have found no significant difference between AD patients and unaffected individuals [203].

Vitamin E has been known to suppress brain lipid peroxidation and reduce both Aβ levels and amyloid plaque deposition significantly in Tg2576 mice, if the vitamin E is administered early enough when the disease is just beginning to develop. If the vitamin E supplement is given later, after the amyloid plaques have formed, a reduction in the oxidative stress in the mice brain is observed, but with no significant effect on the symptoms of amyloidosis. This suggests that the increased lipid peroxidation in brain precedes the amyloid plaque deposition in the Tg2576 mice model and that the brain oxidative damage is an early event in AD [204]. Therefore, there is a strong indication that

therapy with antioxidants, to target the oxidative stress should be initiated in the earliest stage of the disease.

Vitamin E can reduce the Aβ induced cell death in rat hippocampal cell culture [205]. Vitamin E has also been known to reduce the cell death in PC12 cultures against both Aβ40 and Aβ42 insults [206]. There is an increase in protein oxidation and formation of ROS when Aβ42 is added to a culture of primary rat embryonic hippocampal neurons, but adding vitamin E to the culture can inhibit the neurotoxicity it causes. Vitamin E does not inhibit the Aβ42 fibril formation though [207].

Aβ is also known for inflicting toxic effects on vascular cells [208]. When Aβ40 was added to a culture of SMC there was an increase in ROS, such as O_2^-, which is an indicator of oxidative stress. Pre-treating the cells with vitamin E gave protection against the Aβ40 assaults, indicating that vitamin E can reduce significantly the Aβ mediated cytotoxicity on SMC [209].

The effect of antioxidants on cystatin C (L68Q) toxicity and has not yet been determined.

1.12.3. Tramiprosate

Sulfated glycosaminoglycans (GAG) are component of proteoglycans, which are known to promote the transition of Aβ from random coil to β-sheet rich conformation, and in that way contribute to the fibril formation. Proteoglycans are also known to have a role in protecting the fibrillar protein from proteolysis [210]. Tramiprosate (3-amino-1-propanesulfonic acid, 3-APS, Alzhemed^{TM}), is an ionic compound developed to mimic sulfated GAG and in that way interacts with soluble Aβ to maintain it in a non-fibrillar form [211].

When tramiprosate is added to cell cultures of mouse hippocampal neurons, which were previously exposed to Aβ42, there is a decrease in the Aβ42-induced cell death and an inhabitation of amyloid deposition. Tramiprosate was found to bind equally well to Aβ40 and Aβ42, and prefer to bind the soluble form of Aβ rather than the fibrillar form [211]. When transgenic mice with FAD mutation where treated with tramiprosate, it resulted in about 30% reduction of the amyloid plaque load in the brain compared to control mice. There was also a dose-dependent reduction in the plasma Aβ level; the reduction was found to be up to 60%. Furthermore, there was up to 20-30% decrease in the cerebral levels of both soluble and insoluble Aβ40 and Aβ42 [211]. Overall, the *in vivo* experiments showed that tramiprosate could effectively hinder the formation of fibrillar amyloid by binding to the soluble Aβ amyloid.

Targeting existing amyloid plaques, as a therapy, could oligomeric Aβ from the plaques, which would increase toxicity. Therefore, it is advantageous to target the soluble Aβ, like Tramiprosate does, and in that way prevent further deposits without affecting the already formed plaques [211].

The exact mechanism of Tramiprosate action is not yet fully known. Most likely the Tramiprosate binds to the Aβ peptide and in that way form an inactive complex. This complex is probably more likely to be effluxed from the brain or catabolised than the soluble Aβ [212]. Tramiprosate can be orally administered, crosses the blood-brain barrier and has a half-life of about 2-4 hours in blood plasma. Interestingly, the half-life was found to be much longer in the brain, or about 14-48 hours [211].

Tramiprosate and the tau protein was looked at, Tramiprosate was shown to promote the aggregation of tau protein inside the cells and although this tau protein aggregation was not shown to be cytotoxic. It needs to be taken into account when considering future use of Tramiprosate as a drug [213].

Tramiprosate has been found to be both safe and well tolerated in *in vivo* studies [214]. There are few side effects following long-term administrations in rats and dogs, mainly mild gastrointestinal effects with high doses [211]. Yet sadly, when the drug went to phase III study with AD patients with mild-to-moderate AD, as a multi-centre, randomized, double-blind, placebo-controlled study, there was not significant reduce of symptoms in AD patients observed [215].

The effect of Tramiprosate on cystatin C (L68Q) toxicity has not yet been determined.

2. Aim of this study

The aim of this study is to further explore the mechanism of toxicity excerted on cells by amyloid materials such as Aβ protein and variant form of cystatin C. The emphasis will be on the dose dependent toxicity of the variant cystatin C on both cerebral vascular smooth muscle cells and neuronal like cells and testing of potential protective agents. Both cell viability and morphological changes in cells will be explored. Interactions between different lengths of Aβ peptides and varient cystatin C will be explored regarding differences in toxicity. Therapeutic agents, which have showed promise for reduction of Aβ toxicity on both smooth muscle cells and neuron like cells will be tested against variant cystatin C toxicity.

3. Materials and methods

3.1. Human cerebral smooth muscle cells (SMC)

Primary human cerebral vascular smooth muscle cells isolated from a healthy human brain vascular tissue were purchased (Sciencell). These cells are cryopreserved at second passing of the culture and have characterized by immunofluorescence to be both alpha-SMC actin and desmin positive to ensure a homogeneous SMC culture.

3.1.1. Culturing SMC

Human cerebral SMC were grown in a specific SMC medium (Sciencell), supplemented with 2% fetal bovine serum (FBS) (Sigma-Aldrich), 1% penicillin/streptomycin (Sigma-Aldrich) and a 1% concentration of SMC specific growth supplements (Sciencell) designed for optimal SMC growth *in vitro*.

The cells were cultured in T25 culture flasks (Falcon) at 37°C and 5% CO_2. Cell cultures close to confluence were trypsinised with 0.25% trypsin in phosphate buffered saline (PBS) solution, and subcultured in the ratio 1:3. This was done four times and the SMC cells were then cryopreserved and kept in liquid nitrogen while awaiting further use. Freezing-medium consisted of 95% FBS and 5% dimethyl sulfoxide (DMSO). These human cerebral SMC are primary cells and therefore should not be passaged too often.

Prior to use, the frozen cells are quickly warmed to 37°C in a water bath, and centrifuged (Sorvall RT legend) at 100 x *g* for 3 min. The supernatant was discarded and the pellet suspended in fresh culture medium, to remove the DMSO and ensure better cell growth. Cells were cultured in a T25 flask (Falcon) or on 98 whole culture plates (Nunc).

3.1.2. Poly-L-lysine coating

To ensure better adherence of the SMC, it was necessary to pre-coat the culture flasks and plates with poly-L-lysine (Sigma-Aldrich). The culture vessels was incubated with 0.1 mg/mL poly-L-lysine solution for 15 min at room temperature and then rinsed three times with sterile dH_2O. The flasks or plates were then left to dry under a ultra-violet light to reduce the risk of contamination.

3.2. PC12 cells

The PC12 cell line is isolated from rat pheochromocytoma [216] and has proven to be a useful model as neurons in research, since they are highly responsive to nerve growth factor (NGF) that induces them to differentiate into a sympathetic neuron-like cells.

3.2.1. Culturing PC12 cells

PC12 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). On arrival, the PC12 cells were subcultured and when the cells reached about 70% confluence they were passaged in the ratio 1:3. Cells were trypsinated with 0.25% trypsin in PBS for few minutes then fresh medium added and the cells divided up into new collagen coated culture flasks. Subculturing was carried out every 4-7 days. When not needed, subcultures were cryopreserved in liquid nitrogen in a freezing medium consisting of 50%(v/v) RPMI-1640 medium, 40%(v/v) FBS and 10%(v/v) DMSO.

For use, the cells were quickly brought to 37°C in a water bath and centrifuged at 100 x *g* for 3 min and the cell pellet suspended in fresh medium.

3.2.2. Collagen coating

Since PC12 cells adhere poorly to a plastic surface, it was necessary to coat both the culture flasks and plates. Collagen I (Gibco), from rat tail, was diluted to the final concentration of 50 µg/mL in 0.02% acetic acid and added each culture vessel. After 1 hour incubation time at room temperature the flasks or plates were rinsed thrice with PBS. Culture flasks/plates were either used immediately or kept at 4°C until later.

3.2.3. Differentiation of PC12 cells

To differentiate the PC12 cells into a neuron-like state, NGF (Calibochem), isolated from a mouse submaxillary gland, was used. Following NGF addition to the culture the cells stop dividing and start to send out long protrusions that are responsive to electric stimuli [216]. 10 µg were dissolved in 400 µL of sterile dH_2O giving a stock solution with the final concentration of 25 µg/mL and stored at -80°C. PC12 cells were exposed to NGF in the concentration of 50 ng/mL of culture medium (1% FBS, 0.1% penicillin/streptomycin in RPMI-1960). Throughout the experiments the presence of NGF is kept constant to maintain dendrite formations.

3.3. Isolation of cystatin C amyloid material

A process to isolate the variant form of cystatin C from amyloid deposits in patient´s brain tissue has been developed [217], based on a method published by Pras et al. [218] with a few modification to adapt it to cystatin C.

Post mortem 1 cm thick coronally cut brain section from HCHWA-I patient‘s brain and stored at -80°C, were allowed to thaw over night at -4°C, and then weighed and placed on ice. The leptomeningens were carefully removed with tweezers and homogenized for 2 min in 2 mL volume of saline solution (75 mM NaCl + 0.05 mM benzamidin HCl, pH 7.6) to grind up the tissue for better protein extraction. To extract the saline soluble proteins from the tissue, the volume was increased to

20 mL and the tissue homogenized further for 3 min. All homogenizations were done on ice to prevent protein degradation. The solution was then centrifuged at 10.000 x *g* for 30 min. The substrate contained the saline soluble proteins. An aliquot was collected for further analysis while the remainder of the solution was discarded. Since the amyloid is insoluble in saline solution, it still remained as precipitate. The saline wash of the tissue was repeated 8 times or until the tissue was free of saline washable proteins.

For isolation of amyloid material, the tissue pallet was further homogenized in 20 mL of double distilled water (ddH_2O) and centrifuged at 100.000 x *g* for 1 hour in an ultra high-speed centrifuge. This first fraction contained salt from the previous saline extractions, but the preceding ddH_2O fractions contained solubilised amyloid material from the tissue sample. Although amyloid is not a water soluble material, the lower strength of internal interactions allows the material to be partially solubilised in double distilled water. The supernatant from these fractions were collected and lyophilized. The finished product was a fluffy white material, which was kept for further characterization.

3.4. Characterisation of amyloid cystatin C material

3.4.1. Protein assay

For evaluation of protein concentration in each fraction of extraction, we used a protein assay described by Bradford [219]. This assay is based on an absorbance shift when red form of coomassie reagent binds to proteins, and forms a stabilized blue derivative. The absorbance can be measured at 595 nm. Bradford´s coomassie protein assay is only linear over the range of 2 µg/mL to 120 µg/mL.

Briefly, 5 µL sample from each fraction was added to 95 µL of dH2O. To that mixture 100 µL of Bradford´s coomassie blue solution was added. The sample was vortexed and left to stand for 10 min at room temperature while the chemical reaction was allowed to take place. The sample was then measured in a photospectrometer at 595 nm. For each test, a standard curve from known concentration of bovine serum albumin (BSA) was used as an indicator of the protein concentration of each fraction sample.

3.4.2. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done according to Laemmli [220]. 12.5% acrylamide gels were cast for the electrophoresis of the amyloid material. 15 µL of each fraction solution was added to a 15 µL of a 2x sample buffer solution, containing 100 mM Tris-HCl, 4% SDS, 20% glycerol, 2% β-mercaptoehtanol, 25 mM EDTA and 0.04% bromophenol blue. The SDS in the sample buffer denatured secondary and non-disulfide-linked tertiary structures of the proteins, and applies a negative charge, in proportion to their mass. The sample was heated to 95°C for a period of 5 min, which was to denature the proteins and help the SDS binding to them. The total volume of 30 µL is added per well in the gel and the

electrophoresis performed at 120 V (BioRad) for 1,5 hours or until the lightest protein had reached the end of the gel. The gels were used for either silver staining or western blotting.

3.4.3. Silver staining

Silver staining is both extremely sensitive, able to detect proteins in the ng range, and simple in execution as well as relatively inexpensive. Proteins in the gel bind the silver ions, and with favorable conditions a build-up renders a visible image made of finely divided silver metal [221].

To begin with, the proteins in the polyacrylamide gel were fixated in 30% ethanol (EtOH) and 10% acetic acid for at least 30 min or overnight, followed with a wash in 20% EtOH for 10 min and a rinse in ddH_2O for another 10 min. This wash was repeated twice to ensure the expulsion of possible interfering compounds. For sensitization the gel was quickly submerged in a sensitization solution (0,02% sodium thiosulfate pentahydrate) and then rinsed with ddH_2O. For silver impregnation, the gel was kept in a 12 mM silver nitrate solution for up to 2 hours. For image development, the gel was submerged into a developing solution (3% sodium carbonat, 0.08 mM sodium thiosulfate, 9 mM formaldehyde) and when a desirable contrast of protein bands had formed on the gel, it was relocated to a stop solution (4% Tris, 2% actetic acid) to halt the image development.

Every step was done with agitation, at room temperature and between each step the gel was rinsed with ddH_2O. This method did given a remarkable good contrast on protein bands as well as little or no background.

3.4.4. Western blotting

Western blotting was done according to Towbin et al. [222]. The proteins were transferred from the electrophoresis gel on to a nitrocellulose membrane in a BioRad system at 400 mA current for 45 min. The blot was immunostained with rabbit anti cystatin C IgG (Dako) diluted 1:400, horse radish peroxidase secondary antibody and color developed with the ECL plus system (Amersham Biosciences)

3.5. Cytotoxicity experiments

To evaluate the cytotoxicity of foreign material such as the extracted cystatin C amyloid or the amyloid β-protein, both the morphology and the viability of the cells were monitored. To study the changes in the morphology, cells were cultured to a confluence in a 48-well plate (Nunc) followed with the addition of a cytotoxic factor in different concentrations and the changes in appearance observed and photographed under an inverted phase contrast microscope (Leica). A luminescence-based cell viability test was used to evaluate cell viability based on a luciferase oxidation of luciferin by the aid of ATP [223]. Cells were cultured closed to confluency in a 96-well plate (Nunc), followed by the addition of the cytotoxic material in different concentrations. After predetermined incubation times the cells

were lysated and the ATP concentration in each well measured in a luminometer (Berthold Orion L Microplate Luminometer).

3.5.1. Cytotoxic effects on SMC

For cell viability measurements, human cerebral SMC were cultured in 96-well plates (Nunc) with the total volume of 200 µL SMC medium in each well. At confluency, the medium was reduced to 180 µL and 10 µL of the cytotoxic agent, solubilised in ddH_2O, added at 20x the desired final concentration. Then another 10 µL of sterile ddH_2O were added to bringing the final volume per well to 200 µL. The cytotoxic agents were either the cystatin C amyloid isolated from HCHWA-I patient´s brain tissue, Aβ40 or Aβ42. Lyophilized Aβ42 and Aβ40 were obtained from Dr. Einar M. Sigurðsson (New York University). The desired final concentration for cystatin C amyloid ranged from 6.25 - 100 µM and the amyloid β-protein was 25 µM. The dilution of the culture medium had no visible effect on the cells. The cytotoxic effect was allowed to accrue over a period of 2 to 4 days and then the cells were processed for the luminescence-based cell viability testing. For each factor, there were quintuplicate of tests preformed and the ATP concentration is presented as an average of all five.

For morphological studies, the human cerebral SMC were grown to confluency in 48-well plates (Nunc) with the final volume per well at 300 µL. The volume was reduced to 270 µL when the cells reached confluence and 15 µL of the cytotoxic agent, solubilised in sterile ddH_2O, added at 20x the final desired concentration. Then 15 µL of sterile ddH_2O was added to bring the final volume back to 300 µL. The cytotoxic effect were evaluated at regular intervals and photographed under an inverted phase contrast microscope (Leica).

3.5.2. Cytotoxic effect on PC12 cells

For cell viability measurements, PC12 cells were grown to about 60% confluency and then the medium changed from 10% FBS to a 1% FBS medium supplemented with 2 nM NGF (50 ng/mL). This reduction of serum concentration is recommended to ensure differentiation of PC12 cells. The cells were allowed 2 days to gain neuron like morphology and then 20 µL of the total 200 µL of medium exchanged for 10 µL of cytotoxic agents solubilised in sterile ddH_2O, either cystatin C amyloid, Aβ42 amyloid or Aβ40 amyloid, at 20x the desired final concentration and 10 µL of sterile. This dilution of the medium did not have any visible effect on the cells. The cytotoxic effects were monitored over 2 to 3 days, and then the cells processed for the luminescence-based cell viability testing. As before, each factor was tested in a quintuplicate of wells and the final ATP concentration presented as an average of the five measurements.

For morphological observance, the cells were allowed to grown to about 40% confluency in 24-well plates (Nunc) with 500 µL of medium per well, supplemented with 1% FBS and 2 mM NGF. Differentiation was monitored for up to 48 hours in a phase contrast inverted microscope (Leica) and to evaluate the cytotoxic effect of cystatin C amyloid material and amyloid beta protein, 450 µL of

medium was replaced by 25 µL of cytotoxic agent, at 20x the desired final concentration, and 25 µL sterile ddH_2O. The cytotoxic effects were monitored and recorded as before.

3.6. Possible therapeutic agents.

Effects of protective factors were evaluated on cell cultures in ether 96-well or 48-well plates (Nunc). Either SMC or PC12 cells were grown to the desired density for cytotoxicity evaluation. PC12 were allowed two days for neuron differentiation prior to testing. The experimental setup was the same as for the cytotoxicity experiments, described above. Before the addition of the cytotoxic factors, the possible protective agent was added to each well in the desired concentration

3.6.1. Humanin

Humanin (Sigma-Aldrich) was solubilised in sterile ddH_2O to a concentration of 1000 µM. For storage, solubilised humanin was kept at -20°C. For testing, for possible protective effect, 2 µL of humanin was added to each well of 96-well plates and 5 µL for 48-well plates, giving the final concentration of 10 µM. This concentration has been shown to protect smooth muscle cells against assault from the variant form of Aβ40 with the Dutch mutation [198].

3.6.2. Alfa-tocopherol (Vitamin E).

Oxidative stress due to amyloid assaults has been demonstrated repeatedly [207]. One of the factors shown to reduce the stress and thereby the toxic effect is alfa-tocopherol also known as vitamin E. Vitamin E is a fat-soluble antioxidant that stops the production of reactive oxygen species. This form of vitamin E was solublised in FBS, since it is not water soluble. This vitamin E enriched FBS was substituted for the regular FBS in the SMC medium, giving the final concentration of either 50 µg/mL or 25 µg/mL of vitamin E. After the addition of vitamin E enriched medium, the cytotoxic agents were added. Each well was compensated with sterile ddH_2O to give a final volume of 200 µL in the 96-well plates, or a final volume of 300 µL in the 48-well plates.

3.6.3. Tramiprosate (Homotaurine, Alzhemed).

Tramiprosate (3-amino-1-propanesulfonic acid) (Sigma-Aldrich), also known as homotaurine and marketed as the drug Alzhamed. It has been used as a potential treatment for Alzheimer's disease since it binds to the soluble amyloid beta protein and can inhibits it´s aggregation [213].

Tramiprosate was dissolved in water in the concentration 8 mM. For cell viability assay in 96-well plates, 10 µL of this solution was added to each well giving the final concentration of 400 µM per well. Tramiprosate was added before the addition of the cytotoxic factors. Each well was compensated with sterile ddH_2O to a total volume of 200 µL. For morphological observance, 48-well plate were used

with, 15 µL of 8 mM Tramiprosate solution in 300 µL total volume to give a final concentration of 400 µM. Freshly solubilised Tramiprosate was used for each experiment.

3.7. Luminescence-based cell viability testing

Determining cell viability by a luminescence-based assay that measures the adenosine triphosphate (ATP) concentration within a cell culture is a very sensitive and accurate assay. The reaction between luciferase and luciferin requires ATP and release energy in the form of luminescence that is proportional to the amount of ATP present. It is possible to detect the ATP in as little as 20 cells/mL and there is a completely linear relationship between the number of viable cells and light output provided that the cells maintain a relatively invariant ATP content. We employed the viability assay develop by Ian Cree and associates [223].

A solution of 10 mM NH_4VO_3 (Sigma-Aldrich); 500 mM Hepes (Sigma-Aldrich); and 0.5% Triton-X100 (Sigma-Aldrich) was used to disrupt the cells and extract the ATP also available. After 50 µL of this reagent was added to each well, containing 200 µL of medium, the plate was incubated at room temperature for 15 min. Then 10 µL of this cell lysate was transferred to a non-transparent white 96-well plate (Thermo) along with 40 µL of 20 mM hepes buffer. Then, 50 µL of the luciferase-luciferin solution were added and luminescence measured in a luminometer (Berthol, Orion L) immediately. The luciferase-luciferin solution consisted of 0.15 mg/mL luciferase and 0.45 mg/mL of D-luciferin, with the luciferase (Kikkoman Corp.) made up in 0.6 M Tris-succinate (Sigma-Aldrich); and 1.25% BSA (Sigma-Aldrich) at pH 7.5 and the D-luciferin (BioThema) in 10 mM Tris(hydroxymethyl)aminomethan (THAM) (Sigma-Aldrich); 185 mM Hepes (Sigma-Aldrich); and 46 mM MgSO4 at pH 7.7. Both the extraction solution and the luciferase-luciferin solution can be purchased readymade form different suppliers, for instance Promega. For measuring the ATP content of the cell lysate, from each well was and 40 µL of 20 mM hepes buffer added. Then, 50 µL of the luciferase-luciferin solution were added and the. ATP standard curve was made from known ATP concentration standards. Plates where ATP extract solution has been added to wells, can be stored at -20°C for up to a month and still give satisfactory ATP measurements [223].

3.8. Statistics

Statistic analysis was performed in SigmaPlot using one-way ANOVA for multiple comparisons a Holm-Sidak method was used. Significance level was set at 0.05. Statistical analyses of each data set are shown in appendix.

4. Results

4.1. Isolation of cystatin C amyloid material from HCHWA-I patients brain tissue samples.

The method for isolation of cystatin C amyloid material has previously been adapted from Pras et al. [217] and has recently been described in details [217]. The source of the amyloid material was frozen HCHWA-I brain tissue.

About 1 g of leptomeningeal membrane was collected from similar section of brain tissue from different individuals with HCHWA-I, homogenisated and the amyloid material extracted. The end product weighs only a few milligrams representing little less than one percent of the weight of the leptomeningeal starting material. Table 1 shows the yield form two typical isolations.

Table 4.1 Recovery of cystatin C amyloid deposits from brain tissue.

	Sample 1	Sample 2
Brain section	40.59 g	46.58 g
Membrane collected	1.0867 g	1.0859 g
Lyophilized amyloid material	8.2 mg	7.4 mg
Deposits weight precentage of membrane collected	0.75 %	0.68%

The first part of the isolation process involved an extensive wash with saline solution to extract all saltwater soluble material out of the tissue sample. To monitor the process of saline soluble protein expulsion, a sample of each fraction was collected and the amount of protein determined as depicted on the left side of figure 4.1.

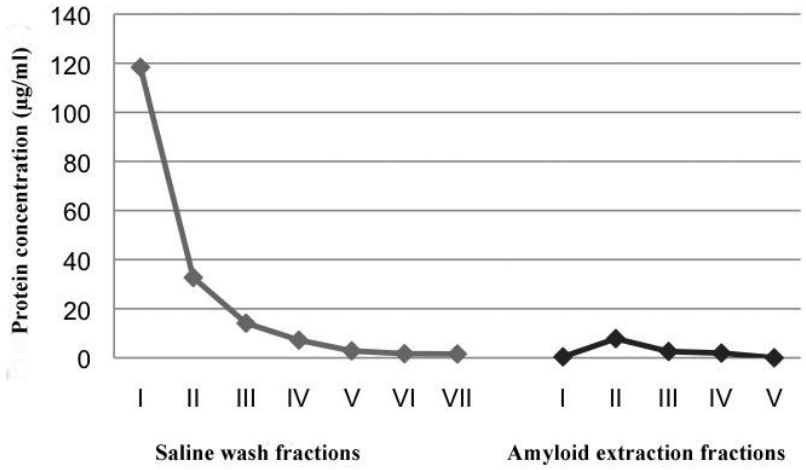

Figure 4.1 Proteins extracted in each isolation fraction. The amount of protein was determined for each extracted fraction. Light grey line on the left represents the seven water fractions. The dark grey line on the right represents the amount of protein in each ultrapure water extraction containing the solubilised cystatin C amyloid. Each data point is in triplicate.

As figure 4.1 shows, most of the saline washable proteins were expulsed in the first few washes. By the fifth fraction, the protein concentration was already under 3 µg/mL and after the seventh fraction it was safe to conclude that there are little to no saline soluble proteins remaining in the tissue sample. Ultrapure water was used for the extraction of amyloid material as seen on the right side of figure 4.1. The first fractions contained no detectable proteins. This was attributed to the salt being washed out of the tissue sample. The second amyloid extraction showed a protein concentration of 7.8 µg/mL and then a steady decline of protein concentration in the succeeding fractions. By the fourth amyloid ultrapure water fraction, the protein concentration was less than 2 µg/mL and by the fifth fraction the protein concentration was below the level of detection. The extractable amyloid deposits from the brain tissue were therefore considered to be exhausted by the fifth fraction.

The ultrapure water fractions, two through five, were collected and lyophilized over a period of few days. The retrieved amyloid material was collected and its weight determined (Table 4.1).

4.2. Characterization of the extracted amyloid material.

Each fraction of the isolation process was subjected to polyacrylamide gel electrophoresis (SDS-PAGE) for protein analysis. Figure 4.2 shows a silver stained 12.5 % acrylamide gel loaded with 10 µL sample of each extracted fraction. The first saline wash fraction shows a dense mixture of proteins being washed out (lane 2). In fraction two and three (lanes 3 and 4), there is still considerable amount of salt soluble proteins extracted, but the succeeding fractions show a considerable decline in protein concentration, ending as relatively protein free (lanes 5 to 9).

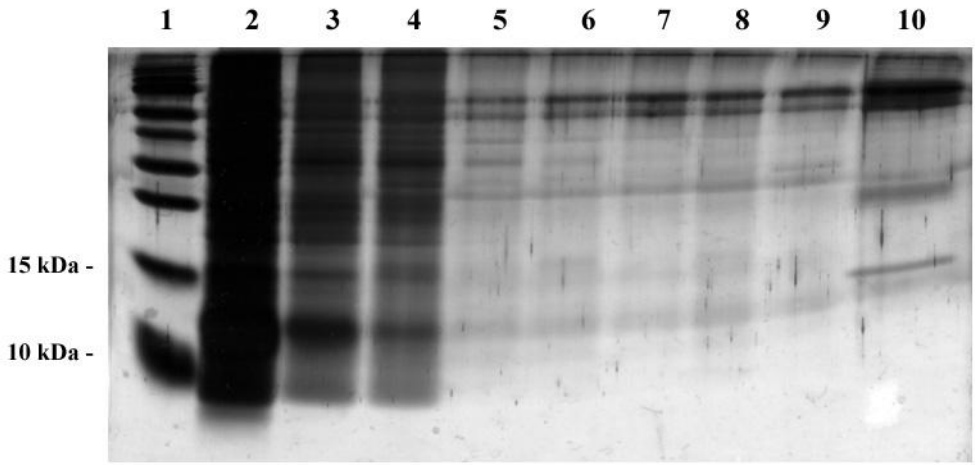

Figure 4.2 Silver staining of saline wash fractions. In the saline wash fraction, the undesired salt water soluble proteins were washed out of the tissue sample. **Lane 1**: Molecular weight markers. **Lane 2**: First saline wash fraction. **Lane 3**: Second saline wash fraction. **Lane 4**: Third saline wash fraction. **Lane 5**: Fourth saline wash fraction. **Lane 6**: Fifth saline wash fraction. **Lane 7**: Sixth saline wash fraction. **Lane 8**: Seventh saline wash fraction. **Lane 9**: Eighth saline wash fraction. **Lane 10**: CSF from a HCHWA-I patient.

We could see that a relatively few washes were needed to render the tissue mostly free of saltwater soluble proteins leaving the saltwater insoluble amyloid material trapped within the tissue sample Ultra pure water was used to solubilise and extract the amyloid material from the tissue. Figure 4.3 shows the silver stained acrylamide gel loaded with 10 µL sample of each ultra pure water fraction. As already indicated by protein quantification (figure 4.1), there were no detectable protein bands in the first amyloid extraction fraction, whereas the bulk of the proteins were seen in the second fraction. Each consecutive fraction showed considerable decline in protein amount with only few faint silver stained bands detected by the fifth fraction (figure 4.3).

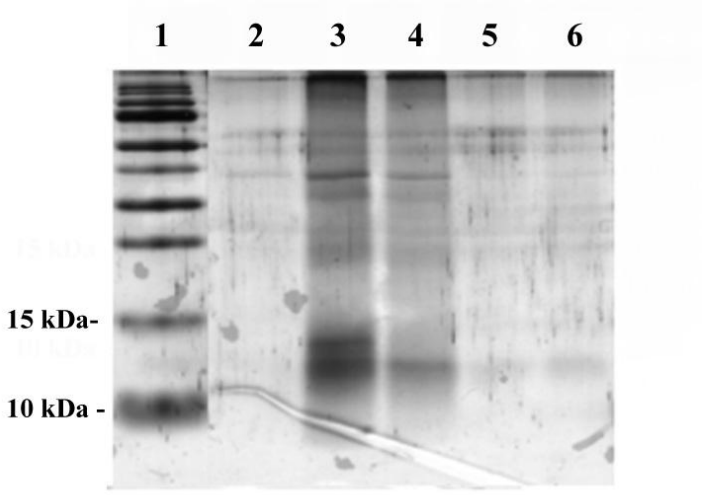

Figure 4.3 Silver staining of amyloid extraction fractions. In the amyloid extraction fractions, the reactive ddH_2O managed to partially solubilise the amyloid material. **Lane 1**: Protein ladder. **Lane 2**: First amyloid extraction fraction. **Lane 3**: Second amyloid extraction fraction. **Lane 4**: Third amyloid extraction fraction. **Lane 5**: Fourth amyloid extraction fraction. **Lane 6**: Fifth amyloid extraction fraction.

Amyloid isolation fractions 2 and 3 (figure 4.3, lane 3 and 4) show a prominent band with apparent molecular weight about 13 kDa, which corresponded to the molecular weight of cystatin C, and another band slightly higher is apparent in fraction 2. These bands diminished by each successive fraction.

Following lyophilisation of the ultrapure water fractions, a white fluffy material remained. To analyze this lyophilized material a small amount from each fraction was solubilised in ultrapure water and the electrophoresed protein were silver stained (figure 4.4). These samples represented the solibilised mixture of cystatin C amyloid material as it was introduced to the cell cultures for toxicity testing.

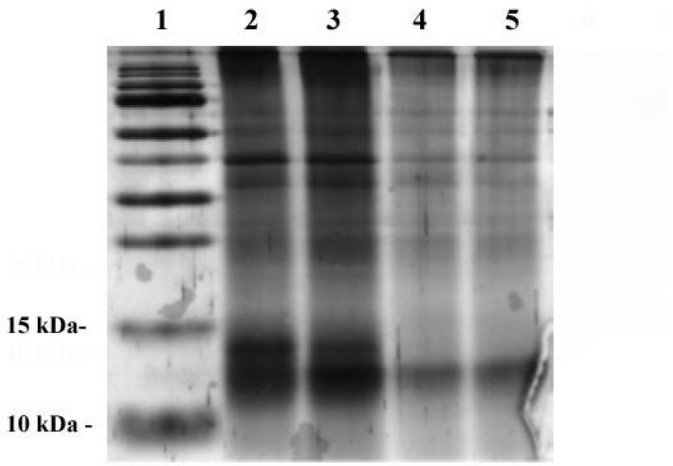

Figure 4.4 Silver staining of lyophilised ultra pure water fractions. A small piece of the lyophilized material collected after the isolation process was solubilised, electrophoresed and silver stained. **Lane 1**: Molecular weight markers. **Lane 2**: Second amyloid extraction fraction. **Lane 3**: Third amyloid extraction fraction. **Lane 4**: Fourth amyloid extraction fraction. **Lane 5**: Fifth amyloid extraction fraction.

Two prominent protein bands with molecular weight around 13 kDa were prominent among the extracted proteins, especially outstanding in fraction 2 and 3 (figure 4.4, lanes 2 and 3), corresponding to the molecular weight of cystatin C. Ladder of bands are seen with higher molecular weight, possibly representing aggregated form of cystatin C.

To verify the extent of cystatin C in the ultrapure water fraction, a sample of each isolation fraction was electrophorised and electroblotted from the acrylamide gel to nitrocellulose membrane and immunostained for cystatin C.

Figure 4.5 shows that cystatin C immunoreactive material was found in the saline wash fractions, representing the innate cystatin C protein that is soluble under physiological conditions.

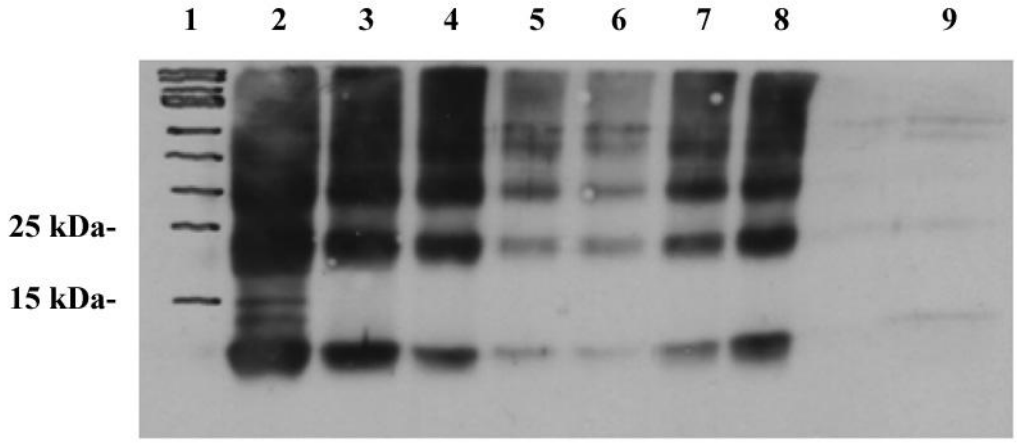

Figure 4.5 Cystatin C detected in the saline wash fraction by Western blotting. Each amyloid extraction fraction was electrophorised and then immunoblotted against cystatin C and developed with ECL plus developer. **Lane 1**: Molecular weight markers. **Lane 2**: First saline wash fraction. **Lane 3**: Second saline wash fraction. **Lane 4**: Third saline wash fraction. **Lane 5**: Forth saline wash fraction. **Lane 6**: Fifth saline wash fraction. **Lane 7**: Sixth saline wash fraction. **Lane 8**: Seventh saline wash fraction. **Lane 9**: CSF from a HCHWA-I patient.

The ultra pure water extraction (figure 4.6) showed no cystatin C immunoreactivety at first (figure 4.6, lane 2) as expected since the corresponding silver stained gel (figure 4.3, lane 2), which also showed no protein bands in this fraction. This was due to salt being washed out of the tissue sample. Extensive cystatin C immunoreactivity is apparent in the successive ultra pure water fractions, both in the low molecular weight bands, around 13 kDa and the higher bands, that show a pattern that correspond roughly to multiple of the molecular weight for cystatin C, suggesting oligomeric forms of cystatin C.

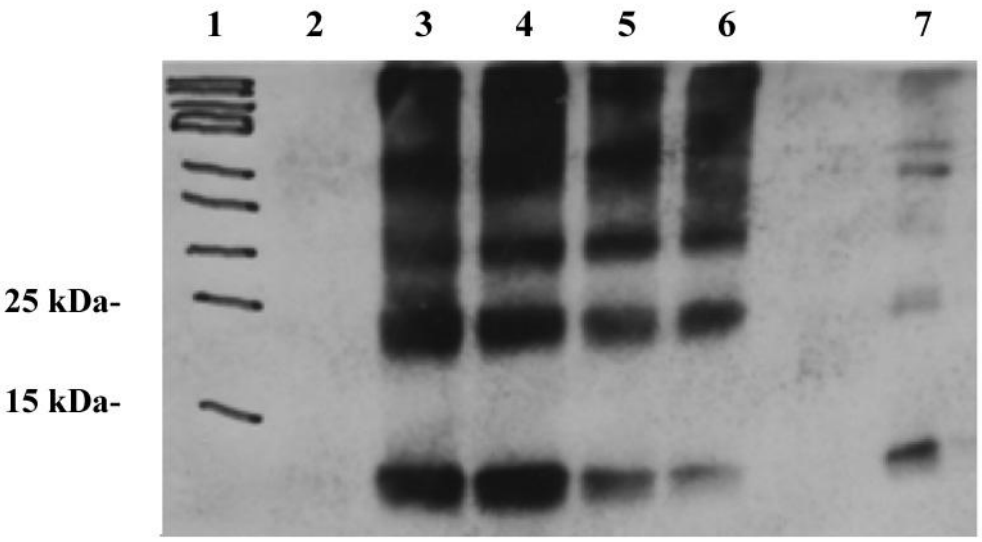

Figure 4.6 Cystatin C detected in the pure water fraction by Western blotting. Sample from each amyloid extraction fraction was electrophorised and blotted against cystatin C and developed with ECL plus. **Lane 1**: Molecular weight markers. **Lane 2**: First amyloid extraction fraction, containing mainly salts. **Lane 3**: Second amyloid extraction fraction. **Lane 4**: Third amyloid extraction fraction. **Lane 5**: Fourth amyloid extraction fraction. **Lane 6**: Fifth amyloid extraction fraction. **Lane 7**: CSF from a HCHWA-I patient.

Moreover, this proved to be compatible to the silver staining of the same fractions suggesting that all or most all of the detectable proteins are cystatin C immunoreactive. This implies that the bulk of the protein in the ultra pure water fraction is cystatin C in different state of polymerization or aggregation (figure 4.3).

4.3. Further immunoblotting of the amyloid material

Other material is known to accumulate in amyloid deposits. For Aβ amyloid in Alzheimer´s disease, amyloid associated proteins include apolipoprotein E, apolipoprotein J and p-component. The amyloid material isolated from HCHWA-I brain tissue, was blotted and immunostained for each of those amyloid associated proteins and no immunoreactivity was detected. This strengthens our conclusion that the amyloid material isolation using this process is relatively pure.

4.4. Toxicity of solubilized cystatin C amyloid material on SMC.

To further investigate the toxic effect of solubilised cystatin C amyloid materials, different concentration were added to SMC in culture. Molar concentration was determined from cystatin C monomeric molecular weight and the impact of the cystatin C amyloid was tested in concentrations ranging from 0 µM to 75 µM (figure 4.7).

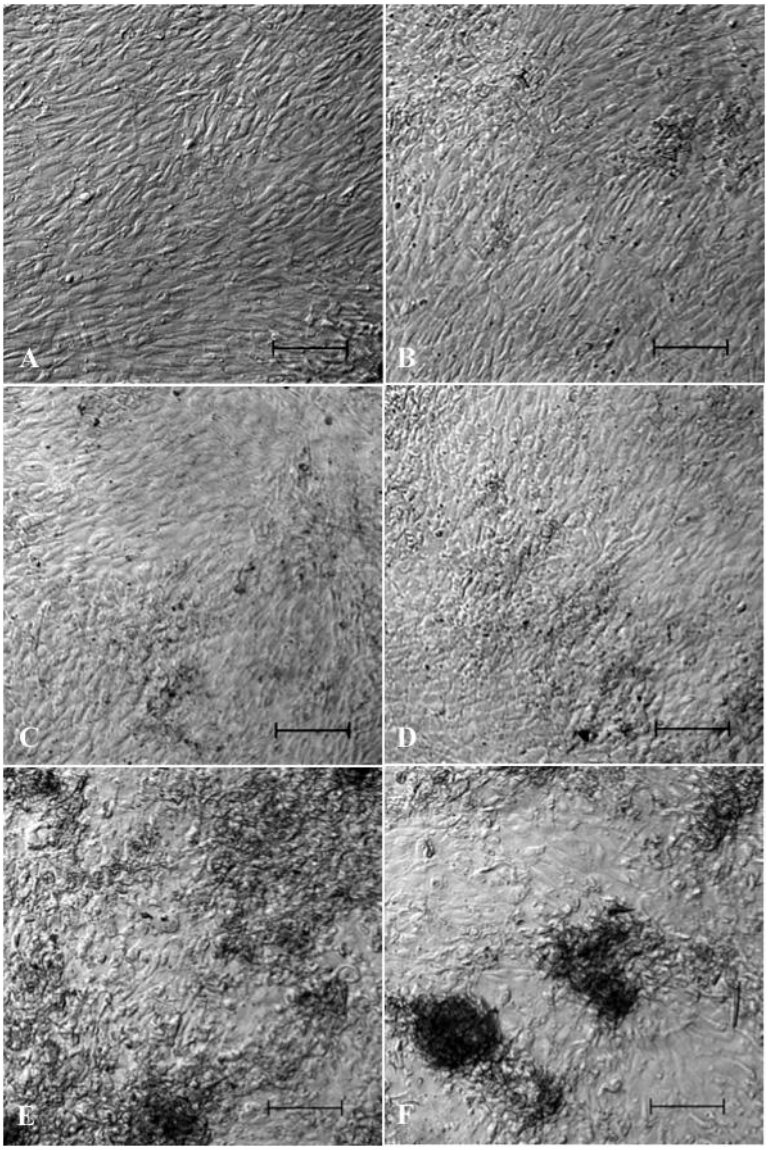

Figure 4.7 The quantitative toxic affect of solubilised cystatin C amyloid on SMC. Solubilised cystatin C amyloid was introduced to SMC culture in various concentrations and incubated for 2 days at 37°C and 5% CO_2. **A**: Shows a SMC culture without any toxic agent. **B**: A sister SMC culture with 6.25 µM cystatin C amyloid. **C**: SMC culture with 12.5 µM cystatin C amyloid. **D**: SMC culture with 25 µM cystatin C amyloid. **E**: SMC culture with 50 µM cystatin C amyloid. **F**: SMC culture with 75 µM cystatin C amyloid. The bar represents 100 µm.

Figure 4.7A shows a confluent primary cerebral vascular SMC culture. The culture forms a tight monolayer of typical spindle shaped cells following serum deprivation.

In figure 4.7B, we see culture incubated with 6.25 µM of solubilised amyloid cystatin C. No drastic toxic effect could be seen, although some clumping of the cells was apparent. Slight brown color forms where the cell mass is denser, caused by refraction of light.

In figure 4.7C, the concentration of solubilised amyloid cystatin C was increased to 12.5 µM resulting in little more clumping of cells that became much more prominent at 25 µM (figure 4.7D) with the cells losing the elongation formation which could be seen in untreated cells (figure 4.7A).

Figure 4.7E shows the effect of 50 µM of solubilised amyloid cystatin C. Here the cytotoxic effect was quite visible with the cell clumping together in piles, leaving cell free patches in the cell layer.

In figure 4.7F, we see the toxic reaction of 75 µM of solubilised cystatin C amyloid. The effect was drastic with dying cells clumping together in large piles, leaving large open spaces between the piles and much fewer cells in the culture.

Figure 4.7 shows obvious qualitative toxic effect of solubilised amyloid cystatin C. The toxic agent causes the cells in culture to lose their elongated spindle form, decrease in size and aggregate into clumps. The cell population diminished with the growing concentration of solubilised cystatin C amyloid in the culture.

Having this visual observation of the toxic effect of solubilised amyloid cystatin C on SMC culture, there was a need for a more accurate quantitative approach. The most sensitive method to determine cell viability at our disposal is the ATP-luciferase luminescence cell viability assay. The SMC were grown in 96 well plates and when they reached confluence, they were incubated with solubilised cystatin C amyloid over a period of 2 days. Then the cells were lysed and the ATP concentration determined in the lysate from each well giving a good indication of cell viability.

ATP concentration diminished in a linear fashion with the increase of solubilised cystatin C amyloid concentration (figure 4.8), an indication of a concentration dependent cytotoxicity of solubilised cystatin C amyloid. A concentration of 12.5 µM cystatin C amyloid reduced the cell viability by half, while a 50 µM cystatin C amyloid concentration brought the cell viability down to 90%. A 100 µM concentration of cystatin C amyloid was sufficient to annihilate most of the SMC in the cell culture.

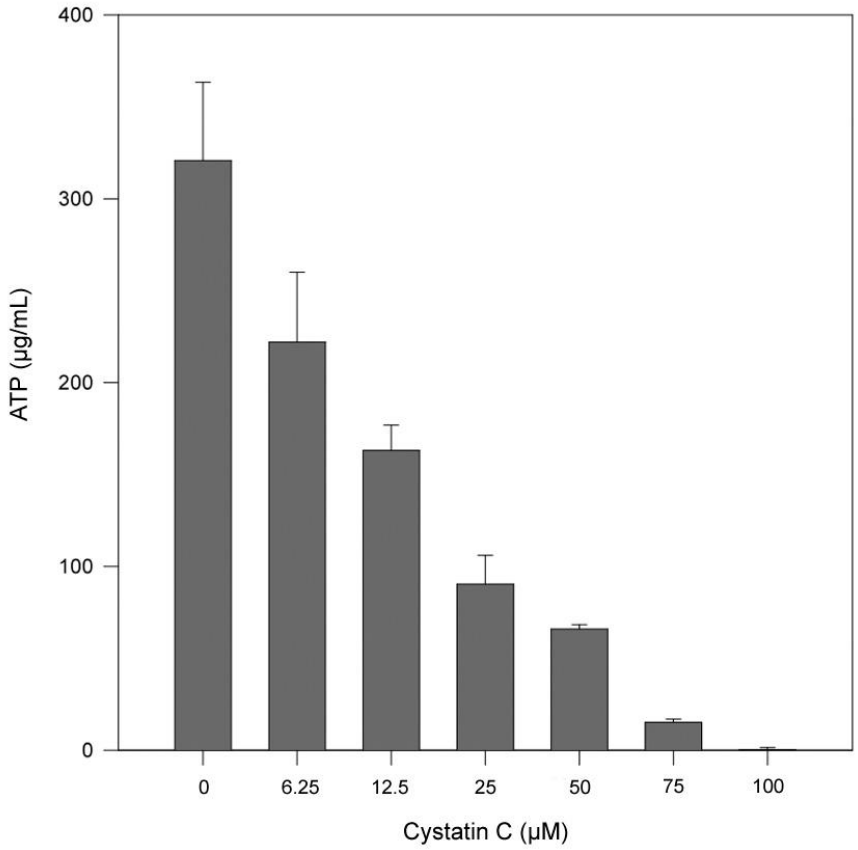

Figure 4.8 Quantification of the toxic effect of solubilised amyloid cystatin C on SMC. SMC were cultured in 96-well plate, grown to confluence before introducing different concentration of solubilised cystatin C amyloid and incubated for 3 days at 37°C and 5% CO_2. The toxic effect of solubilised cystatin C amyloid was linear between the tested range of 0 – 100 µM. Error bar represents mean +/- SEM, n = 3.

For further analysis of the cytotoxic effect of solubilised cystatin C amyloid, the concentration of 12.5 µM was chosen. This concentration gives sufficient toxic effect, 50% cell death in three days, while conserving limited cystatin C amyloid at our disposal. Furthermore, choosing a concentration with a medium toxicity increases the possibility for detecting moderate protective effects from a therapeutic agent that otherwise might go unnoticed.

4.5. Toxicity of amyloid β protein on SMC.

Alzheimer´s disease (AD) is a more common and much better studied amyloid condition than HCHWA-I. The amyloid forming protein in AD is the amyloid β protein (Aβ), which is prone to aggregate and deposits in the neurophil of the brain but also to some extend in the cerebral vasculature similar to cystatin C amyloid. The cytotoxic affect of both amyloid agents can be quite potent, causing SMC death.

To test the toxic effect that the Aβ protein has on SMC culture in our set up, the Aβ protein was resuspended in ultrapure water and added to the culture. There are two common forms of toxic Aβ protein, Aβ42 and Aβ40, where the number corresponds to the length of the peptide. The cytotoxic effect of the Aβ protein was monitored after a three-day exposure and photographed (figure 4.9).

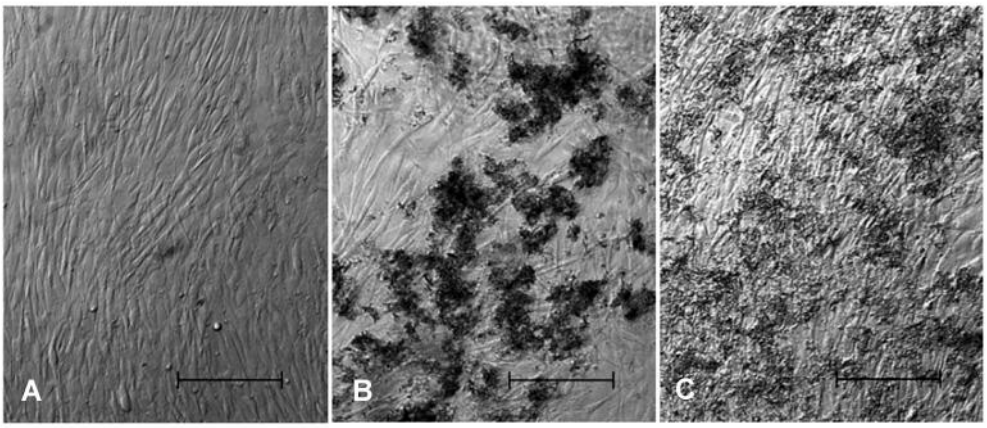

Figure 4.9 Cytotoxic effect of Aβ40 and Aβ42 on SMC culture. Different amyloid agents were introduced to confluent SMC culture and incubated for 3 days at 37°C and 5% CO_2. **A**: Untreated SMC culture. **B**: Sister culture of SMC incubated with 25 µM Aβ40 peptide. **C**: Another sister culture of SMC incubated with 25 µM Aβ42. SMC were incubated with toxic agent for 3 days. Bar represent 200 µm.

Figure 4.9 shows healthy SMC culture with and without addition of toxic Aβ peptide. When amyloid β protein was added to the SMC culture, the toxic effects were similar to the cystatin C amyloid toxicity. The SMC began to clump together and lose their characteristic spindle shape. Their cell bodies appear to shrink in size and their numbers diminished resulting in cell free patches in the monolayer indicating cell death during the 3 days incubation period. In the scientific literature, an Aβ concentration of 25 µM has become a standard concentration for cytotoxic testing of Aβ protein.

To confirm and quantify cell death, the ATP-luciferase cell viability assay was preformed. Confluent cells in 96-well plates were incubated with either Aβ42 or Aβ40, incubated for 2 days and the ATP concentration determined in the cell lysate for each well (figure 4.10).

Solubilised cystatin C amyloid was added to a culture in the concentration 12.5 µM, or half the molar concentration used for the Aβ proteins. After 3 days of incubation with 12.5 µM solubilised cystatin C amyloid, cell survival was estimated to be about 57% ($p<0.001$) based on ATP level, 47% with 25 µM of Aβ40 ($p<0.001$) and around 76% with 25 µM Aβ42 ($p=0.002$). Aβ42 seemed to be less toxic to SMC than Aβ40, yet it was significantly toxic compared to untreated cultures. These findings are in line with the aggressive nature of HCHWA-I.

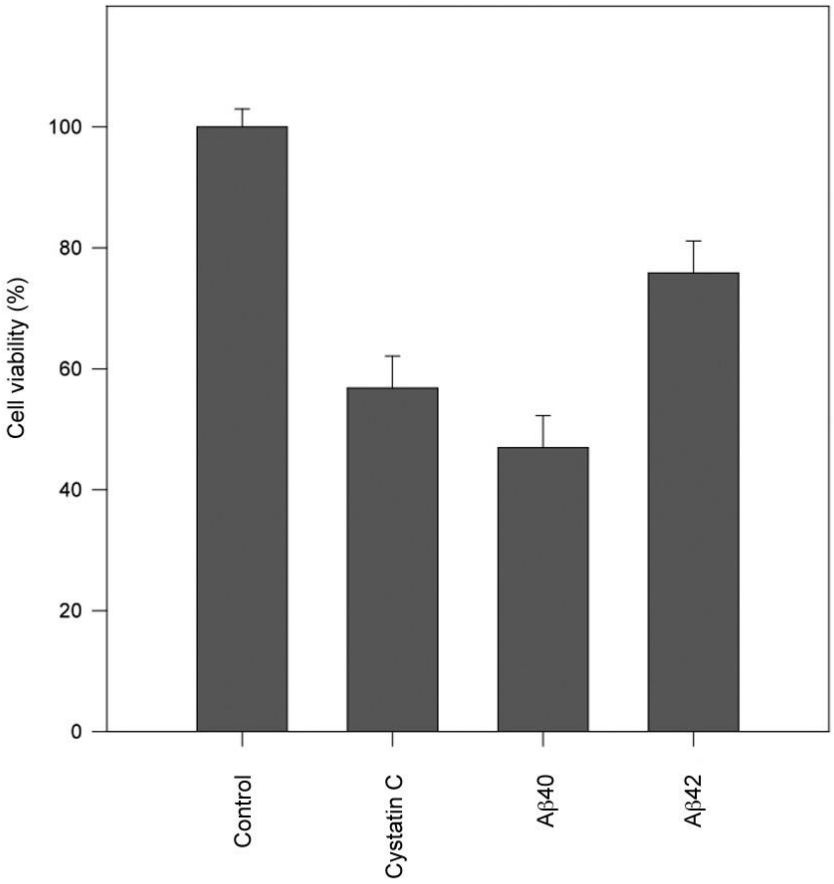

Figure 4.10 Cytotoxic affect of Aβ proteins and cystatin C amyloid on SMC in culture. Confluent cultures of SMC were incubated with one of three amyloid agents, 25 µM Aβ40, 25 µM Aβ42 or 12.5 µM solubilised cystatin C amyloid. After 2 days at 37°C and 5% CO_2, the ATP concentration was determined in the lysate of each cell culture well. A significant toxicity was found for all three amyloid agents. Data are presented as mean +/- SEM, n = 7.

4.6. Synergistic effect of amyloid β and cystain C amyloid.

The toxicity of Aβ40 and Aβ42 was tested together with solubilised cystatin C amyloid to test for possible synergy. The combination of each of the Aβ peptides with the cystatin C amyloid material showed some additive toxic effect, although far from adding up to the total toxicity of both agents separately (figure 4.11). When cystatin C amyloid was added to the cell culture, cell viability dropped to 77% after 2 days incubation ($p=0.001$). If Aβ42 was added, the drop was to 77% ($p<0.001$) and 57% for the Aβ40 ($p<0.001$). When a combination of 12.5 µM cystatin C amyloid and 25 µM Aβ42 is added to SMC culture, the cell viability reduction to 64% ($p<0.001$), which is lower than cystatin C amyloid or the Aβ42 did separately. When a combination of 12.5 µM cystatin C amyloid and 25 µM Aβ40 was added to a SMC culture, the cell viability dropped to 50% ($p<0.001$), which again is lower than cystatin C amyloid or Aβ40 did separately.

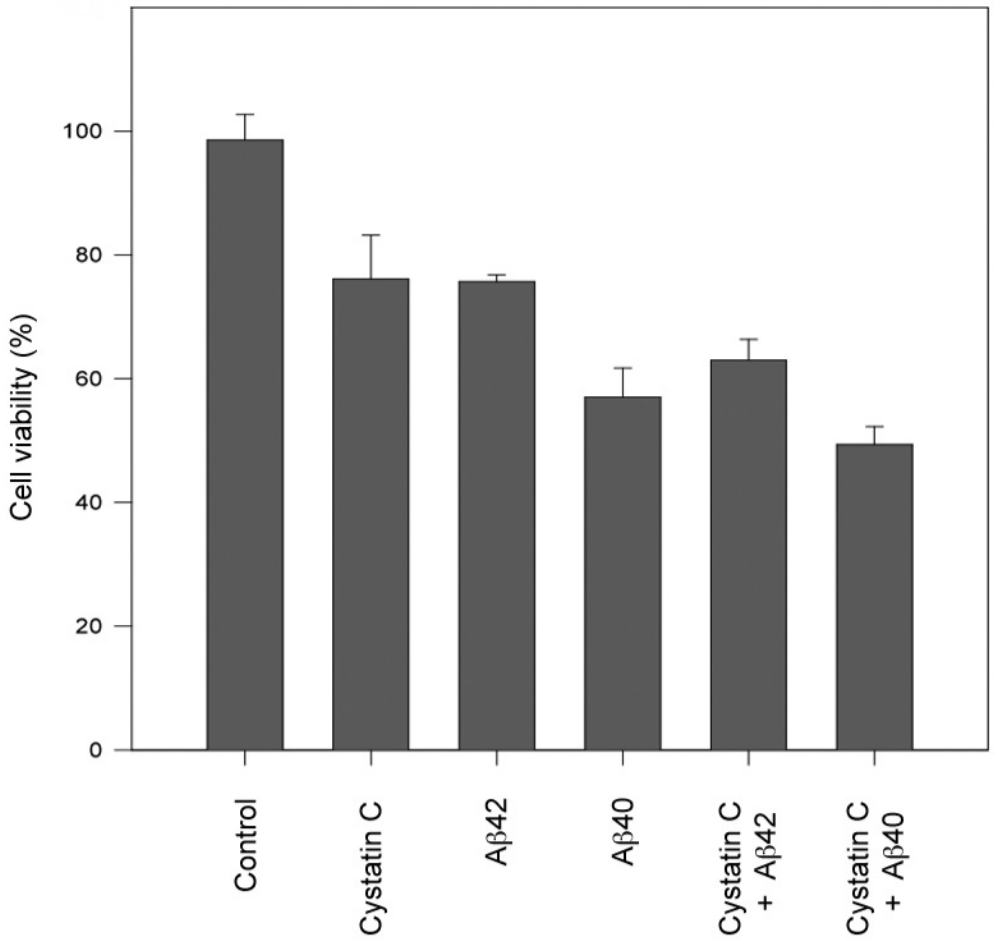

Figure 4.11 Toxic affect of Aβ40, Aβ42 and solubilised cystatin C amyloid on SMC in culture. SMC culture were introduced to solublised amyloidogenic agent, either 12.5 µM cystatin C, 25 µM Aβ40, 25 µM Aβ42 or a combination of either 12.5 µM amyloid cystatin C with 25 µM Aβ42 or a combination of 12.5 µM cystatin C amyloid and 25 µM Aβ40. After incubation for 2 days at 37°C and 5% CO_2, the cells were lysed and the ATP concentration measured with ATP-luciferase cell viability assay. Data represents mean +/- SEM, n = 5.

4.7. Theraputic agents

4.7.1. Humanin

Humanin is a 26 amino acid peptide found to protect neurons against Aβ cytotoxicity. Moreover, humanin was reported to protect SMC against toxic assaults from the Dutch variant of the Aβ protein (Aβ40D). There, the humanin protection was proven to be affected in the concentration range of 100 nM – 10 µM.

10 µM humanin was added to an untreated SMC culture as well as to a SMC culture treated with 12.5 µM solubilised cystatin C amyloid. The effect of this was both monitored under a bright field microscope as well as by measuring ATP concentration for each cell culture using luciferase cell viability testing.

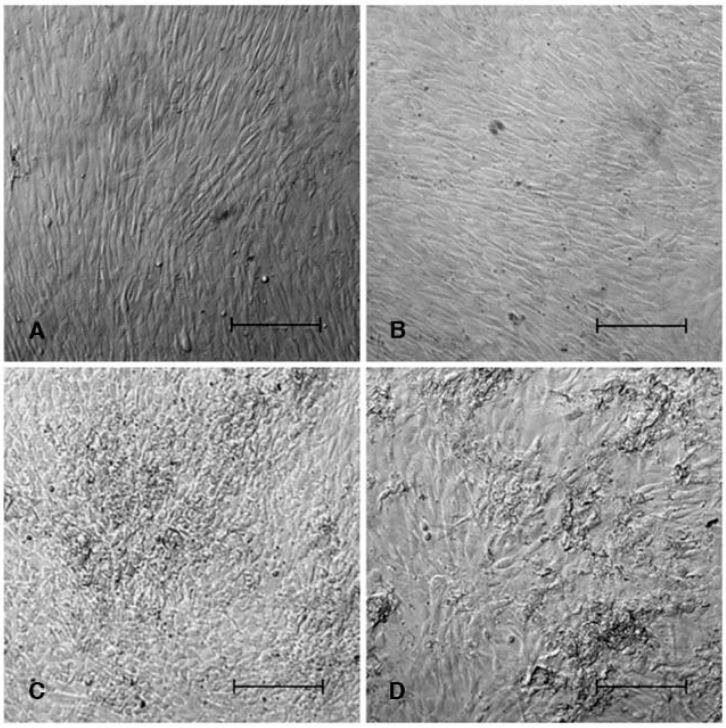

Figure 4.12 Effect of humanin against cystatin C amyloid assault on SMC. A: An untreated SMC culture. **B**: A sister SMC culture with the addition of 10 µM humanin. **C**: A sister SMC culture with the addition of 12.5 µM cystatin C amyloid. **D**: A sister SMC culture with the addition of 10 µM humanin before the addition of 12.5 µm cystatin C amyloid. SMC were incubated for 2 days at 37°C and 5% CO_2. Bars represent 200 µm.

When humanin was added to a SMC culture, there were no visible changes to the cell culture (figure 4.12B). The SMC retained their typical elongated spindle shape (figure 4.12C). When solubilised cystatin C amyloid was added to the cell culture, a characteristic toxic reaction was seen with clumping and shrinking of the cells (figure 4.12C). When 10 µM humanin was added to the SMC culture prior to the 12.5 µm of solubilised cystatin C amyloid, the characteristic toxic effect could still be seen. Humanin did not appear to have any protective effect against the solubilised cystatin C amyloid. To quantify the results the cell viability was determined with the ATP-luciferase assay (figure 4.13).

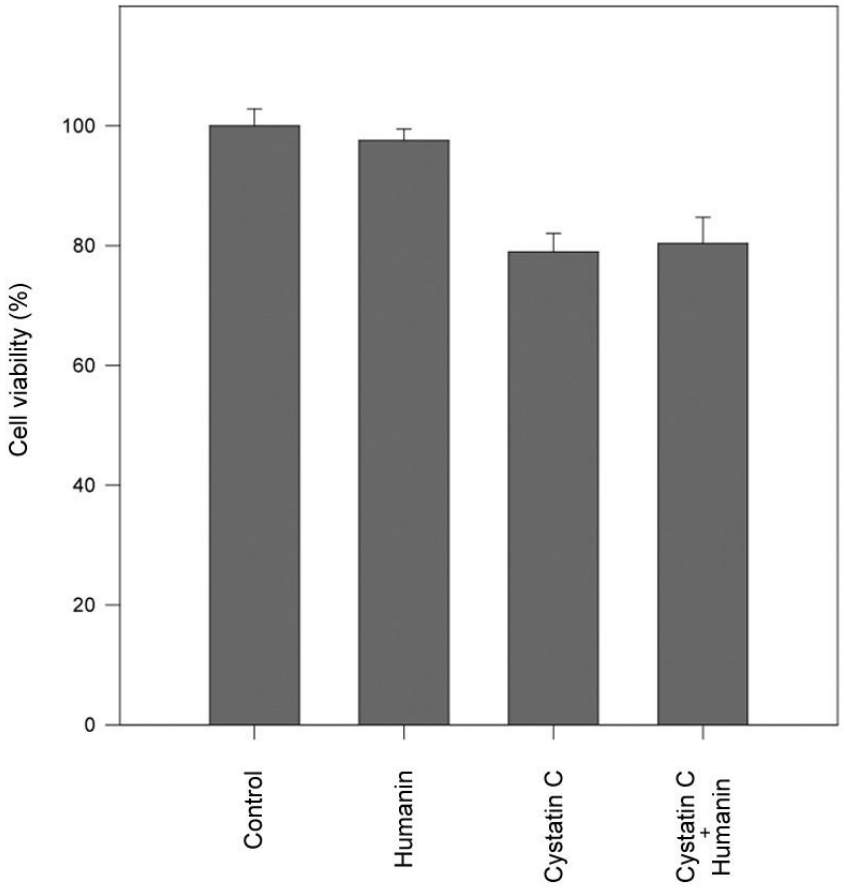

Figure 4.13 Effect of humanin on SMC without or with solubilised cystatin C amyloid. SMC were either untreated or subjected to 10 µM humanin (HN), 12.5 µM cystatin C amyloid or both. Cells were incubated for two days at 37°C and 5% CO_2, and then the ATP quantified in the cell lysate to determine cell viability. Error bars represent mean +/- SEM, n = 5.

The addition of humanin to a SMC culture did not have a significant impact on the cells viability. When treated with solubilised cystatin C amyloid the cell viability dropped significantly, to 79%

($p<0.001$). With the presence of 10 µM humanin in the cystatin C amyloid treated cell culture the viability was still only 80% indicating no significant protective influence of the humanin.

Humanin has proven itself effective in protecting SMC from amyloid beta protein toxicity. This experiment was repeated in order to verify this effect against Aβ40 protein toxicity in our system.

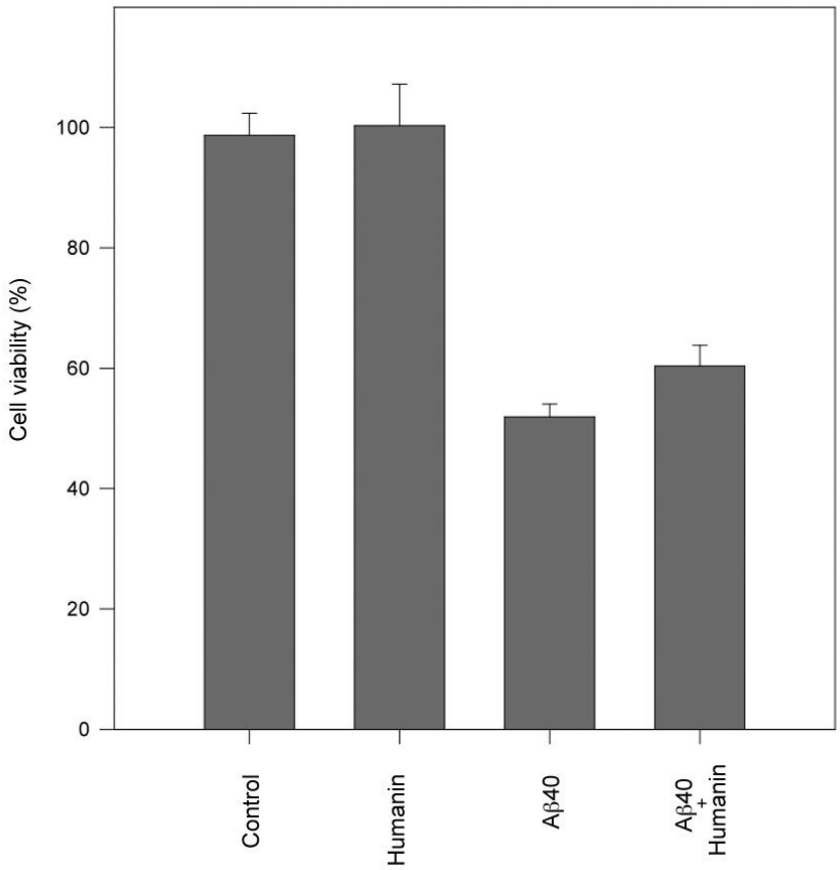

Figure 4.14 Effect of humanin against solubilised Aβ40 amyloid on SMC. SMC were either untreated or subjected to 10 µM humanin, 25 µM Aβ40 amyloid or both. Cells were incubated for two days at 37°C and 5% CO_2, lysated and the ATP concentration for each culture was measured with luciferase luminescence cell viability assay. Error bars represent mean +/- SEM, n = 5.

As was seen before, the addition of 10 µm humanin did not have a significant effect on the smooth muscle cell culture. When introduced to the Aβ40, in the concentration of 25 µM, the cell viability dropped to 51% significantly ($p<0,001$). By adding 10 µM humanin to the cell culture prior to the addition of Aβ40 protein, seemingly there was a partial inhibition of the amyloid toxicity on the SMC. When the data was statistically analyzed with a t-test between Aβ40 and when HN is added alongside the Aβ40, there is a slight protection which proved to be significant ($p<0,018$).

4.7.2. Vitamin E (α-tocopherol)

One mechanism proposed for Aβ cytotoxicity is oxidative stress within the cell. The use of antioxidants as a therapeutic agent has been well documented. The use of 50 µg/mL vitamin E has been reported to protect for both neurons and SMC against Aβ protein.

The addition of vitamin E to a SMC culture did not seem to have any significant effect. When 12.5 µM of cystatin C was added to a sister culture, the characteristics of cytotoxicity were visible, both the clumping and rounding up of cells along reduction in cell size. When the 12.5 µM solubilised cystatin C amyloid was added to a SMC culture containing 50 µg/mL vitamin E enriched medium, there was no visible protection.

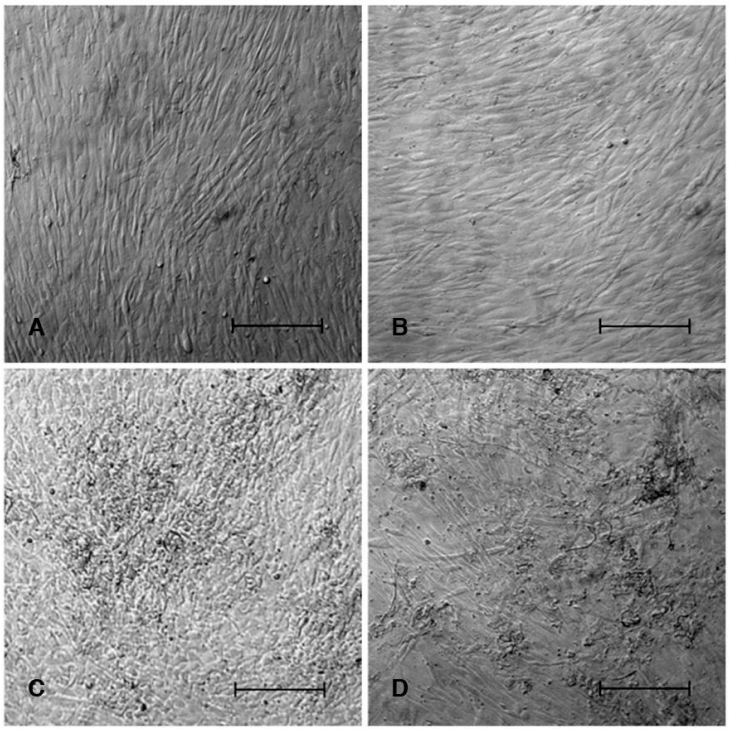

Figure 4.15 Effect of vitamin E against cystatin C amyloid toxicity on SMC. A: Culture of untreated SMC. **B**: Sister culture with the addition of 50 µg/mL vitamin E. **C**: Sister culture with the addition of 12.5 µM cystatin C amyloid. **D**: Addition of 50 µg/mL of vitamin E before the addition of 12.5 µM cystatin C amyloid to a SMC culture. Cells were incubated for 2 day at 37°C and 5% CO_2. Bars represent 200 µm.

Vitamin E has been used in the concentration 50 µg/mL on cell cultures. High concentrations have also been shown to be toxic to cell cultures [209, 224]. To accurately measure the viability of the cells, the ATP content of cell lysate from each culture was measured with luciferase cell viability assay. Two concentrations of vitamin E were tested, 50 µg/mL and 25 µg/mL.

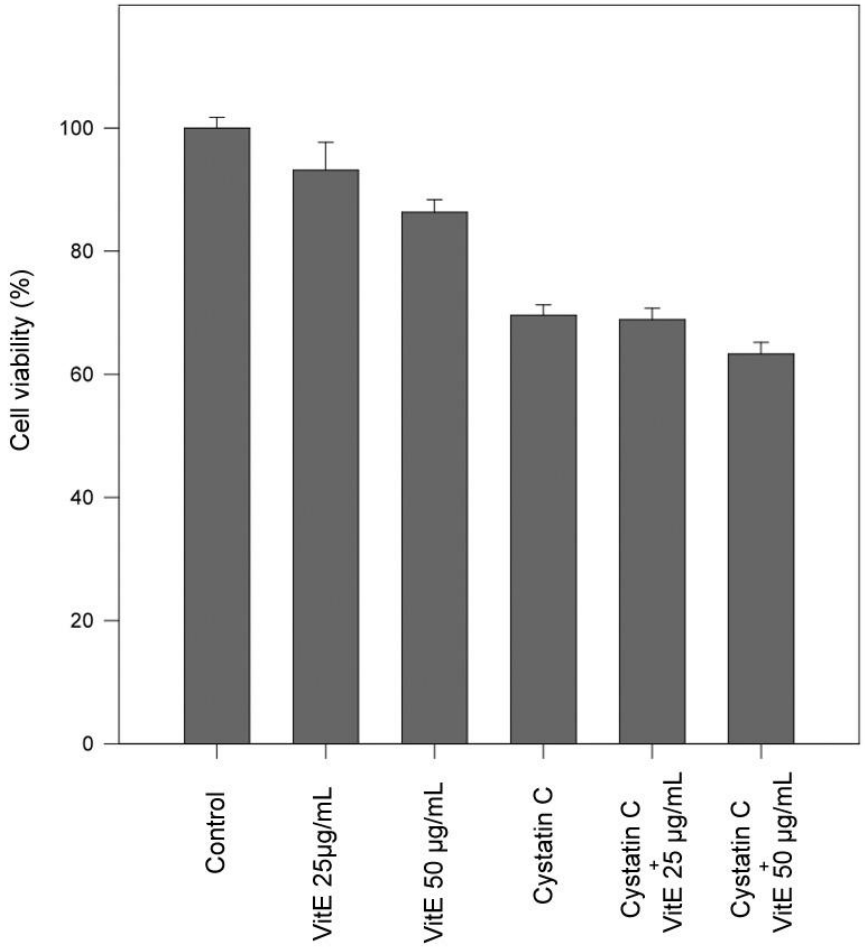

Figure 4.16 Effect of vitamin E against cystatin C amyloid assaults on SMC. SMC cultures were either untreated or treated with vitamin E, 12.5 µM solubilised cystatin C amyloid or both. Vitamin E was tested in two concentrations, 25 µg/mL and 50 µg/mL. Cells were incubated for 2 days at 37°C and 5% CO_2. Cells were lysated and ATP concentration measured for each cell culture. Error bars represent the mean +/- SEM, n = 5.

Addition of 50 µg/mL vitamin E showed no visible toxicity on SMC. With the sensitivity of the ATP-luciferase viability test there was still a slight yet significant lowering of cell viability ($p<0.001$) down to 86%, indicating that 50 µg/mL had some toxic affect. When the dose of vitamin E was lowered to 25 µg/mL concentration, there was not a significant lowering of viability detected. With the addition of solubilised cystatin C amyloid the cell viability dropped to 70% ($p<0.001$). The addition of vitamin E, in either concentration, to the cell culture prior to the addition of 12.5 µM solubilised cystatin C amyloid, showed no significant change in cell viability compared to cystatin C amyloid toxicity alone. Vitamin E does not seem to have a significant protective effect on SMC against cystatin C amyloid assaults

under these conditions. Rather, a high concentrating of vitamin E alone, i.e. 50 µg/mL, can have cytotoxic effect on the SMC.

4.7.3. Tramiprosate

Tramiprosate (3-amino-1-propanesulfonic acid, 3-APS) is a drug marketed for AD patients under the name Alzhemed, and it is thought to halt the polymerisation process of the amyloid protein, preventing the aggregate fibril formation and therefore cytotoxicity. We were interested in seeing if tramiporsate could also prevent the polymerisation of solubilised cystatin C amyloid fibril and thereby protect against its cytotoxic effect.

Tramiprosate was added to SMC culture in the concentration 400 µM along with 12.5 µM solubilised cystatin C amyloid. This concentration of tramiprosate has been used to protect neurons against Aβ toxicity. Addition of 400 µM tramiprosate to a SMC culture (figure 4.17B) did not have any visible effect on the cell culture. The cells maintained their elongated spindle form, like their untreated sister cell cultures (figure 4.17A). By adding 12.5 µM cystatin C amyloid to a cell culture containing 400 µM tramiprosate, similar toxic effect was observed as with 12.5 µM cystatin C amyloid alone (figure 4.17C, 4.17D).

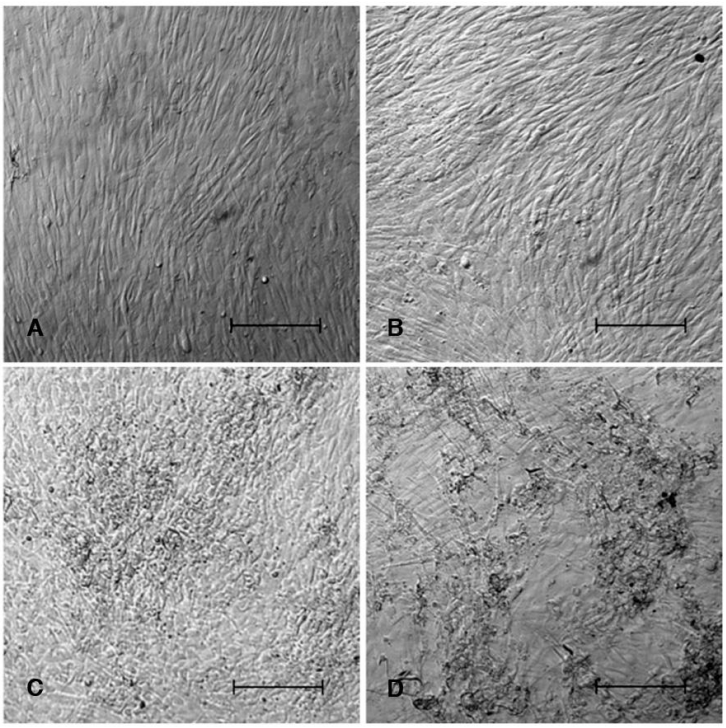

Figure 4.17 Effect of tramiprosate against cystatin C toxicity on SMC. A: Culture of untreated SMC. **B**: SMC culture with added 400 µM tramiprosate. **C**: SMC culture with addition of 12.5 µM cystatin C amyloid. **D**: SMC culture with addition of 400 µM tramiposate and 12.5 µM solubilised cystatin C amyloid. Cells were incubated for 2 days at 37°C and 5%CO_2. Bar represents 200 µm.

The results were quantified by ATP-luciferase assay. Viability in each cell culture was determined, after 2 days of incubation, by measuring ATP concentration in the cell lysate. Addition of 400 µM tramiprosate to SMC culture had no significant effect on the cells viability compared to untreated SMC (figure 4.18). The addition of 12.5 µM cystatin C amyloid had a significant drop in cell viability, to 75% ($p<0.001$). The addition of 12.5 µM solubilised cystatin C amyloid to the cell culture already containing 400 µM tramiprosate the drop in cell viability was 77% ($p<0.001$), which did not significantly reduce the toxic affect compared to the addition of 12.5 µM cystatin C amyloid alone. Tramiprosate did not show any signs of therapeutic effect against cystatin C amyloid toxicity on SMC.

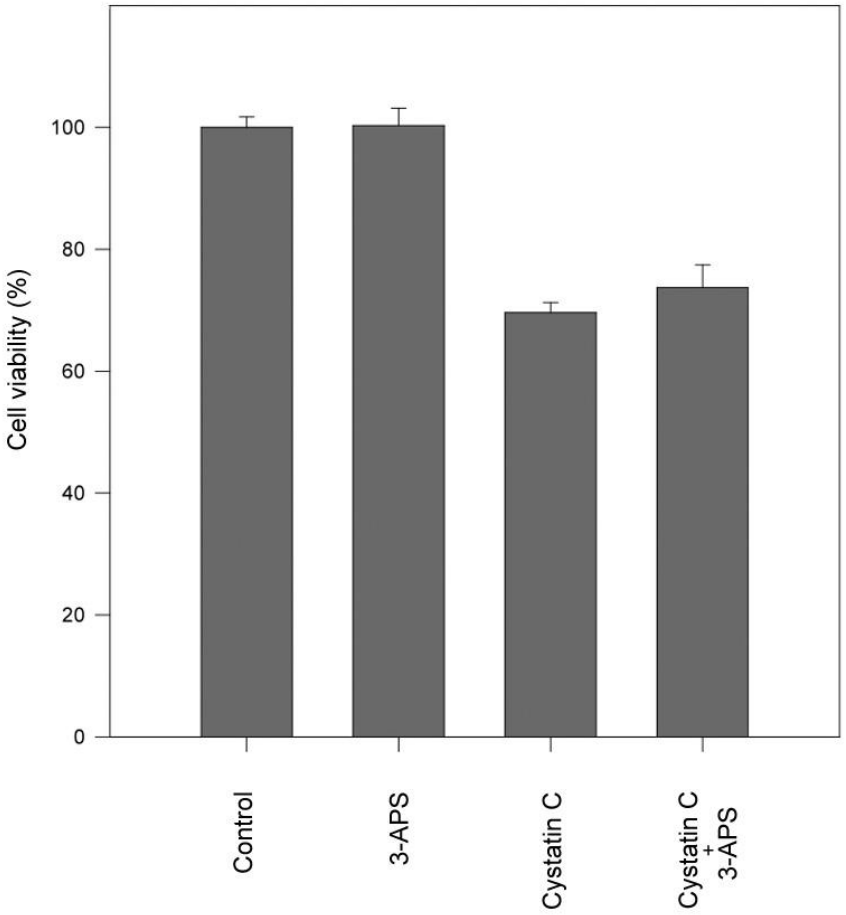

Figure 4.18 Effect of tramiprosate against cystatin C amyloid toxicity on SMC. SMC were either untreated or treated with 400 µM tramiprosate, 12,5 µM cystatin C amyloid, or both. The cells were incubated over a period of two days at 37°C and 5% CO_2, lysated and the ATP concentration in each culture measured with luciferase viability assay. Error bar presented as mean +/- SEM, n = 4.

4.8. Toxicity of cystatin C amyloid on PC12 cells

The neurotoxicity of the amyloidogenic Aβ protein is very well established. However, the equally amyloidogenic L68Q form of cystatin C has not been reported to be toxic to neurons. In HCHWA-I patients the amyloid accumulation is mainly confined to the arterial walls of the brain although cystatin C amyloid is seen to infiltrate into the neurophil from heavily laden vessel, but without any obvious neurotoxic reaction. To test if solubilised cystatin C amyloid is toxic to neurons in culture we used PC12 cells. The PC12 is a rat pheochromocytoma cell line, which responds to nerve growth factor (NGF) by differentiating into sympathetic neuron-like cells, making them a convenient model for neurons in culture.

4.8.1. Differentiation of PC12 cells

PC12 cells were differentiated with NGF in the concentration 50 ng/mL. The cells reacted immediately after the addition of NGF (figure 4.19). One hour after the addition of NGF the PC12 cells had started to sprout their first dendrite (figure 4.19B). A couple of hours later, the cells had flattened and were forming multiple dendrites. Six hours into the differentiation process the PC12 cells could be seen with branched dendrites that already formed connections to their neighboring cells and at 24 hours the cells were connecting over a greater distances with neuron nodules forming on the dendrites. Finally, at 48 hours of differentiations, some PC12 cells had formed an intricate net of dendrites connected to multiple cells. With more time, this dendrite net would thicken, but this time period was deemed sufficient for the cytotoxic experiments.

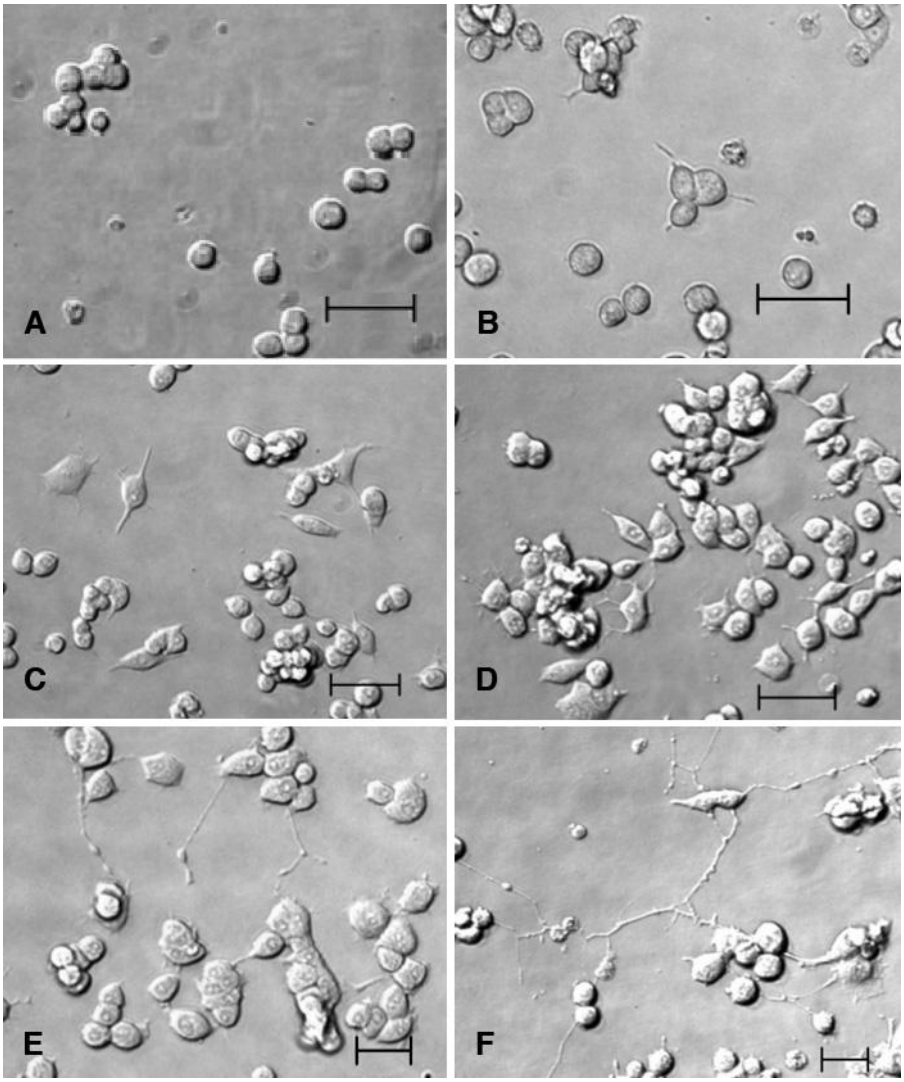

Figure 4.19 Differentiation of PC12 cells. To differentiate the PC12 cells 50 ng/mL of NGF was added to the culture medium and the cells incubated for 2 days at 37°C and 5% CO2. **A**: Undifferentatiated PC12 cell. **B**: After 1 hour of incubation with NGF. **C**: After 3 hours of incubation time. **D**: After 6 hours of incubation. **E**: After 24 hours of incubation. **F**: After 48 hours of incubation. Bar is 10 µm. Cells were incubated at 37°C and 5% CO_2.

4.8.2. Toxicity of cystatin C amyloid on PC12 cells

To test the effect cystatin C amyloid might have on neurons the PC12 cells were grown to confluence, incubated with NGF for 2 days, and then treated with the amyloidogenic cystatin C amyloid material and cultured further for 2 days. The solubilised cystatin C amyloid proved to be harmful to the differentiated PC12 cells (figure 4.20). With the addition of 6.25 µM cystatin C amyloid the PC12 cells

started to clump together. This clumping can be seen increasing with higher concentrations of 12.5 µM cystatin C amyloid, and cell-free areas become detectable at 25 µM concentrations.

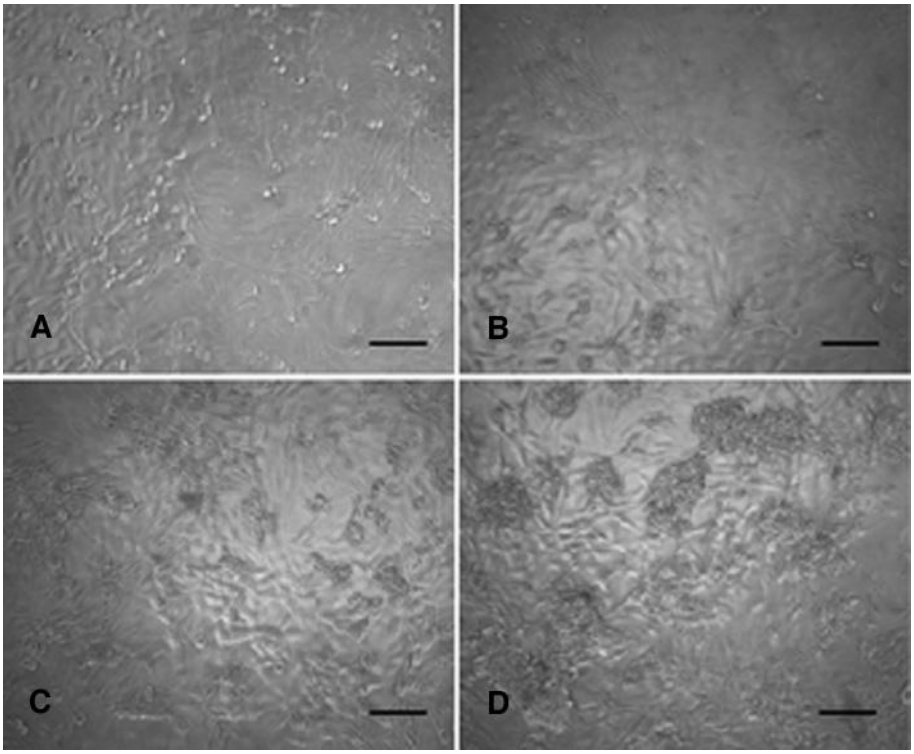

Figure 4.20 Effect of cystatin C amyloid on a PC12 cells in culture. A: Untreated differentiated PC12 cell in culture. **B**: Differentiated PC12 cell culture treated with 6.25 µM cystatin C amyloid. **C**: Differentiated PC12 cell culture treated with 12.5 µM cystatin C amyloid. **D**: Differentiated PC12 cell culture treated with 25 µM cystatin C amyloid. Cells were incubated over three days in 37°C and 5% CO2, lysated and ATP concentration measured. Bar represents 30 µm.

The viability of the PC12 cells introduced to different concentrations of cystatin C amyloid was determined with the ATP-luciferase assay (figure 4.21). The ATP concentration in an untreated cell culture was around 36 µg/mL in each well. This concentration had dropped to about 10 µg/mL with treatment of 12.5 µM cystatin C amyloid for 3 days. When the concentration of the cystatin C amyloid was increased to 25 µM, the ATP concentration dropped to 2 µg/mL and was hardly detectable when treated with 50 µM cystatin C amyloid.

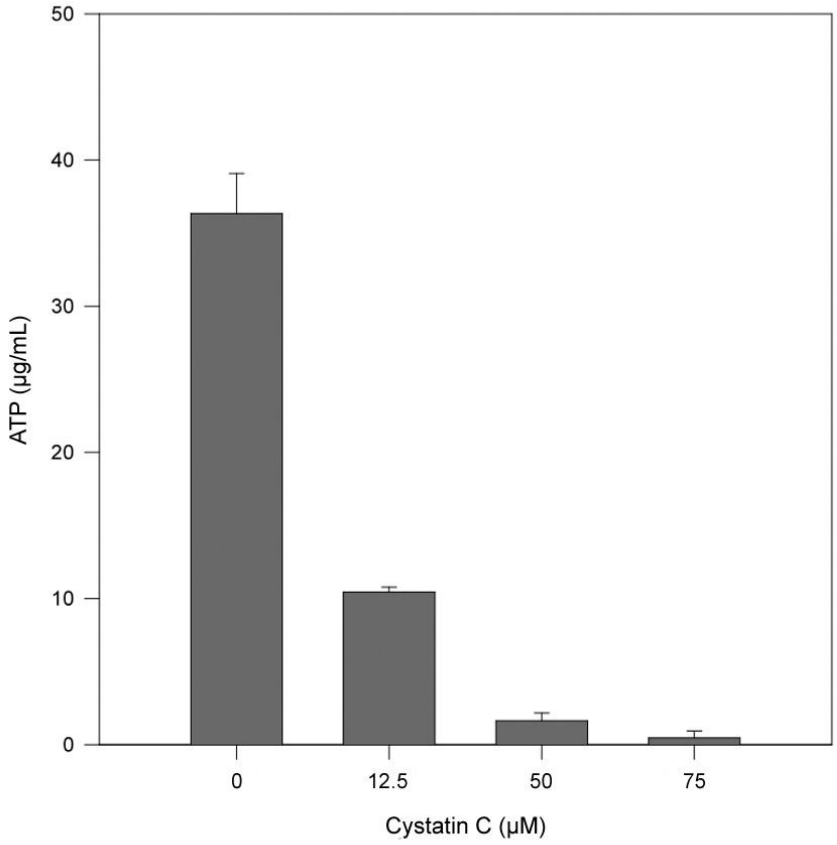

Figure 4.21 Quantification of cystatin C amyloid toxicity on PC12 cells. PC12 cell cultures were incubated with solubilised cystatin C amyloid in different concentrations, 0 - 75 µM, for 3 days at 37°C and 5% CO_2. Viability of the cells was determined by ATP-lusiferase assay. Error bars represent the mean +/- SEM, n = 5.

The cystatin C amyloid material isolated from leptomeningens HCHWA-I patients showed toxic effect in neuron cell culture models, such as PC12 cells. 12 µM concentration dropped the ATP content in the PC12 cell culture down to 10.8 µg/mL from 33.4 µg/mL. There was also a significant drop to 2.3 µg/mL when the cystatin C amyloid material concentration was increased to 50 µM. An increase to 100 µM resulted in 1.1 µg/mL ATP content.

5. CONCLUSIONS

5.1. Isolation of cystatin C amyloid material from brain tissue samples of HCHWA-I patients.

All amyloids show a certain homogeneous and amorphous appearance although different amyloids are known to be made from vastly dissimilar protein segments. Harsh treatment is normally required to solubilise amyloid fibrils, since they are highly insoluble in neutral aqueous media of high ionic strengths commonly used [218]. Therefore, synthetic proteins are generally used for studying the mechanism by which amyloidogenic proteins assemble and cause their cytotoxicity [225]. The amyloidogenic variant of cystatin C, that causes the HCHWA-I, is too large to make synthesis practical. Therefore, a method originally developed by Pras and associates to extract amyloid proteins by solubilisation in pure water [218] was adopted and adjusted for isolating cystatin C amyloid material [217] and successfully used for studying their cytotoxicity [174].

The source of the amyloid material is frozen HCHWA-I brain tissue, obtained from autopsy. This is a very limited resource making good recovery from each isolation, and well-planed experiments, an absolute obligation. Only a few milligrams of amyloid material are recovered from each gram of leptomeningeal membrane.

The first part of the isolation process requires extensive saline washing to abolish all the saltwater soluble material out of the tissue sample, while the amyloid material is retained among the rest of insoluble matter. The total protein concentration drops rapidly in the first few washes and by the fifth fraction is well under 3 µg/mL. Few more washes abolish most of the proteins and little to none is detectable in the seventh saline wash of the homogenized tissue sample.

Each of these saline washes was subjected to SDS-PAGE and silver staining for protein analysis. The first fraction showed a dense mixture of proteins and as the washing proceeded, there was a considerable reduction in the amount and types of protein species. After the first three washes the bulk of the proteins seemed to have washed out and this corresponded well with the protein assay results. Relatively few numbers of washes were needed to render the tissue mostly free of the saltwater soluble proteins leaving our protein of interest, the amyloid cystatin C, in the insoluble tissue mass left behind.

To see if the saline wash was extracting any cystatin C, an electrophorised SDS-PAGE gel, containing those fractions, was electroblotted onto a nitrocellulose membrane and immunostained for cystatin C. With the immunoblotting, we see that there is cystatin C immunoreactive material being washed out in the first fractions, most likely representing the innate cystatin C protein that is found soluble in all organs of the body [32]. The HCHWA-I patients are heterogeneous with the mutant cystatin C gene indicating that there is also a production of healthy cystatin C. Under physiological conditions, native cystatin C is soluble and should wash out in these fractions.

There was a considerable amount of cystatin C being washed out and there was a prominent band around 13 kDa, which corresponded to the monomeric cystatin C molecular weight. A higher band around 25 kDa was also seen, which corresponds to the dimeric form of the cystatin C, and even a distinctive trimer as well. Native cystatin C forms inactive dimers [32, 50]. There are certain factors that

promotes oligomerization of proteins, such as high concentrations or heat [55]. These factors might be at play here. Most likely, this is the normal cystatin C being washed out, but there are higher oligomers than should be expected for the native form. Binding of Aβ and SDS, has been shown to induce oligomerisation when electrophoresis is run [226]. There seems to be an extensive oligomerisation in our gels, but to date there are no antibodies that can distinguish between the native form of cystatin C and the amyloid form with the point mutation. Alternatively, this might represent mutated cystatin C in early stages of aggregation, but still soluble.

After extensive mechanical tear in the homogenizer between each wash, there is a second pulse of cystatin C detectable in figure 4.5. This is thought to be because of a normal cystatin C, which might have gotten caught in the oligomer fibrillar formation and become trapped within the amyloid. The mechanical tearing of the amyloid might have managed to release it.

Solubilitation and isolation of the cystatin C amyloid was started following the extraction of soluble proteins by saltwater. Ultra pure water, which has been shown to render the amyloid material soluble [217], was used. Protein assay showed a protein peak in the second fraction that tapers off into fraction five. All these fractions were collected and further analyzed.

Aliquots of these fractions were analyzed by SDS-PAGE gel and silver stained. There was no detectable protein band in the first fraction as expected, since the tissue sample still contained salt from the saline wash, and corresponds to the results from protein assay. The bulk of the proteins were washed out in the second fraction. A prominent band was detected around 13 kDa in the second and third fractions, corresponding to the molecular weight of the cystatin C. This band appeared as a double. The cystatin C, isolated from amyloid, is known to be truncated of its first 10 amino acids [16, 31]. The slightly higher band might represent the non-truncated form of the cystatin C and the slightly lighter band might represent the truncated. There is no usable anti-body that recognizes the difference between the truncated form and the untruncated forms, so at this point an immunoblotting assay for the difference is not available. By the fifth fraction there was little left of ultrapure water soluble proteins remaining in the tissue sample.

To verify that these bands are in fact cystatin C, the gel was electroblotted for cystatin C immunoreactivety. The Western blot showed that there is no cystatin C immunoreactivity in the first fraction, corresponding with the quantitative protein assay, which showed no protein expulsion in the first ultra pure water wash, and further confirmed by the silver staining that shows little to no staining in this fraction. This fraction washed the salt out of the tissue sample and when the salt is gone, the ultra pure water extraction of the amyloid can begin. In fraction two, we obtained proteins, which showed that one homogenization and wash with ultra pure water is enough to wash the salt out.

The second fraction and the next few fractions contained intensive cystatin C immunoreactivity. The bands corresponding to the molecular weight of cystatin C, 13 kDa, represent a monomeric form of the cystatin C and higher bands of multiples of this molecular weight most likely representing dimeric and higher polymeric forms, especially trimers and a tetramers of the cystatin C but also higher forms of oligomerisation.

Ultra pure water can solublise the amyloid material and draw it out into the water phase from the tissue sample. Each isolation fraction of the ultra pure water with the extracted amyloid was lyophilized. The white fluffy material remaining was then re-solubilised in the ultra pure water. When the lyophilised material was re-solubilised in ultra pure water and the elecrtophoresed and silver stained we saw a clear thick prominent band around 13 kDa, representing the molecular weight of the cystatin C in all fractions, and also the oligomeric forms. The ratio of the oligomeric forms did not seem to have changed from the extraction process, although the concentration of the solution might have become denser, the lyophilisation did not seem to push the oligomerisation process. This sample represented the solublised mixture of cystatin C amyloid material, as it will be introduced to cell culture for toxicity testing.

There are other proteins known to gather in the amyloid formation, like amyloid associated proteins. For the HCHWA-I, immunostaining of brain tissue section have shown that both p-component and apo E are mixed within the amyloid formation. In AD brain, there is an accumulation of p-component, apoE and apoJ within the amyloid β plaques [88, 93, 94]. Too further analyze the end product after the extraction process; the material was immunoblotted for these amyloid associated proteins. The cystatin C amyloid material isolated was not reactive for apo E; apo J; or p-component. This strengthens the conclusion that the amyloid material isolated is relatively pure.

As the final test for purity, a fraction of the isolated cystatin C amyloid material was analyzed by a mass spectrometer (data not shown). Result showed that the material isolated for cytotoxicity experiments is relatively pure form of the variant cystatin C, i.e. L68Q, with no traces of the native cystatin C form. This indicates that the immunoreactive cystatin C being washed out in the saline solution wash is the native form of the cystatin C and the amyloidogenic form is extracted in the ultra pure water extractions. Furthermore, this suggests that the amyloid is only made from the variant form, and native cystatin C does not contribute to the process. The material introduced to the cells in the cytotoxicity experiments is relatively pure form of amyloid cystatin C.

5.2. Cytotoxicity of the amyloid cystatin C

To confirm and further investigate the toxic effect of the solublised cystatin C material, different concentrations, from 0 - 75 µM were added to confluent cultures of human vascular SMC. When cultures with added 6.25 µM of cystatin C amyloid were compared to untreated cultures of SMC, a sign of a toxic effect was detected. There was a slight clumping of cells, and as the concentration was increased, the cell clumping became more apparent. Cells started to lose their morphological structure, such as elongated spindle shape. Patches of cell-free areas were formed where the cells had disappeared. By each increase in the cystatin C amyloid concentration, the effects became more drastic and the number of cell diminished. Thus, there was an obvious quantitative toxic effect of the solublised amyloid cystatin C material. This toxicity caused the cells to lose their morphological character and aggregate into clumps, which dissolved leaving diminished cell population.

To quantify this toxic affect, ATP-luciferase luminescence cell viability assay was utilised on these cell cultures. There is a clear drop in the ATP concentration in these SMC cultures indicating reduction

in viability of the cells. This reduction was dose-dependent, changing with the increased concentration of solublised cystatin C amyloid. The cell viability was reduced by half when treated for 2 days with 12.5 μM cystatin C amyloid. Less than one-fourth of the cell culture was left in 50 μM concentration and fewer than 90% left at 75 μM concentration. All the cells died when a concentration of 100 μM cystatin C amyloid was used. When these concentrations were compared to Aβ concentrations [198], the cystatin C amyloid was found to be more toxic. For the subsequent experiments, where possible protective agents were tested, a concentration was chosen that would have sufficient toxic effect to give the morphological changes, but mild enough to be reversible in an *in vitro* model. In that way, a more moderate protective effect was more likely to be detected. The concentration of 12.5 μM was chosen for the cystatin C amyloid toxicity experiments.

For confirming the Aβ toxicity on SMC, two lengths of the peptide were chosen. Aβ40 is the peptide length most abundantly produced in the body [91] while Aβ42 is the main peptide found in the senile plaques, which characterizes AD [88]. The toxicity of Aβ40 has been tested on SMC [198] but not the Aβ42. The concentration for the Aβ toxicity on SMC cells has been tried with the concentration of 25 μM [198]. In these experiments, the cytotoxic properties of Aβ peptide on human vascular SMC was monitored for 3 days, which had proved to be a sufficient time to produce toxic effect in all amyloid species tested.

When the effects were compared with untreated human vascular SMC the cells started to clump together and lose their characteristic spindle shape, in a compatible manner to the toxic affect seen on the cystatin C amyloid toxicity on the same cells. The cell bodies shrunk in size and their number diminished, leaving cell-free areas.

The cell death in these cultures was quantified by the ATP-luciferase cell viability assay as before. The solubilised cystatin C amyloid was used in half the molar concentration in comparison to the Aβ peptide concentration. After 2 days of toxic effect, the cell viability had dropped down to 57% for the cystatin C amyloid, down to 47% for the Aβ40 but still retained 76% viability for the Aβ42. The Aβ42 showed less toxicity than the Aβ40, which corresponds to other publications. Even half the concentration of the cystatin C still gave a more devastating toxic affect than the Aβ40. This is in agreement with the diseases symptoms since HCHWA-I is a much more aggressive form of brain CAA with early cerebral hemorrhaging compared to Aβ induced amyloid angiopathy.

In other published literature, it has been shown that the native non-toxic cystatin C can bind to the toxic Aβ peptide, and reduces its cytotoxicity [227]. To test whether the variant cystatin C amyloid still possesses this ability, a solution of both cystatin C amyloid and either the Aβ40 or the Aβ42 was added to a cell culture of human vascular SMC. Separately, the cystatin C amyloid, Aβ40 and Aβ42 showed toxic affect on the cells as to be expected. When the cystatin C amyloid was combined with Aβ42, there was an increase in the toxic affect, indicating that there is not a complex forming that reduces the toxic effect of these materials. Rather, both materials were exerting their toxic affect, resulting in a greater loss of cell viability than when these toxic factors were introduced to the cell culture separately.

Same results could be seen when a combination of cystatin C amyloid and Aβ40 was added to the SMC culture. The two amyloids did not seem to form an inert complex as has been reported for the

native cystatin C and the β amyloid. Rather, there is an increase in toxicity, which again indicates that both amyloids are excreting their own toxicity, resulting in even less cell viability compared to the toxicity each amyloid would have inflicted on their own.

This indicates that the point mutation in position 68 is either altering the 3D structure of the cystatin C so the Aβ-cystatin C complex cannot form, or that the variant cystatin C amyloid is too far-gone in oligomerisation that the formation of amyloid complexes between the two species cannot from.

5.3. Search for therapeutic agents

The drugs used today for AD is mainly to increase neurotransmitters in the brain, which might reduce symptoms to a point, but does nothing to reduce the process of the disease itself. For HCHWA-I, there is no treatment. With new understanding of the disease pathway, there are also new openings for new therapeutic drug design.

Humanin has been shown to serve diverse purposes in the body. Its production increases at sites of inflammation or ischemia and is humanin thought to be a part of the body's defense mechanisms. Humanin has been reported to have a protective effect in the concentration of 100 nM – 10 μM against Aβ40 insults on human vascular SMC [198].

When humanin was added to SMC cultures there were no visible changes seen in the cell structure, indicating that humanin is not visibly affecting the SMC on its own. When the solubilised cystatin C amyloid was added to the cell culture, characteristic toxic changes where visible with clumping of cells, reduction in cell body and loss of typical elongated spindle shape. When a mixture of both cystatin C amyloid and the humanin was added on to a SMC culture, these toxic characteristics persisted and they looked no different from culture with only the addition of the cystatin C amyloid. Thus, the humanin does not appear to be protecting the SMC from the solubilised cystatin C amyloid cytotoxicity.

ATP-luciferase cell viability assay was performed on those cells to quantify the results. When the humanin was added to the SMC there was not a significant reduction in cell viability. When the cystatin C amyloid was added to the SMC culture there was a significant drop in cell viability consistent with the toxic effect seen earlier. When a combination of the humanin and the amyloid cystatin C was added to the culture, there was a toxic response and a drop in viability, corresponding to the toxicity seen with just cystatin C amyloid was added to the culture. The addition of humanin with the cystatin amyloid material did not protect the SMC culture as similar studies with Aβ40 toxicity have reported.

To verify the effect of the Aβ40 on the human cerebral SMC, the experiment reported by Nostrand and associates was repeated. Our results show a slight recovery in cell viability against the Aβ40 toxicity on SMC when HN has been added to the mixture.

For AD, there is a strong case being made for oxidative stress as an important factor in the pathology of the disease. Much research has gone into the addition of antioxidants to reduce this stress. Vitamin E is a powerful antioxidant and has been proven to save both neuron cells and SMC from oxidative stress, caused by Aβ peptide [206, 209].

Vitamin E was added to a SMC cultures with no visible effects seen. When the cystatin C amyloid mixture was added to the culture, we noted the characteristic toxic effect. When the cystatin C amyloid was added in combination with vitamin E to the culture, we saw the toxic affect on the cells persisting.

The ATP-luciferase cell viability assay was preformed to quantify the effects on the SMC. Since the concentration of vitamin E was rather high (50 µg/L) a lower concentration (25 µg/mL) was also tried. While addition of the 50 µg/mL showed no visible toxicity, the sensitivity of the ATP luciferase viability test detected a slight, yet significant lowering of cell viability, indicating that a dose of 50µg/mL had some toxic effect. There was not a significant lowering of viability when the concentration was lowered to 25 µg/mL, has been reported that vitamin E on its own, can have toxic effects [224]. This also indicates that the ATP-luciferase viability test is more sensitive than other similar viability test, such as MTT or LIVE/DEAD assay.

When the cystatin C amyloid was added to the SMC culture, we saw the same characteristic toxic effect as always. When a combination of both vitamin E and amyloid cystatin C was added to the culture, not protective effect on the SMC against the assaults of the cystatin C amyloid was apparent under these conditions. Rather, with the 50 µg/mL vitamin E, there seemed to be an addition to the cytotoxicity on the SMC. We saw no protection by vitamin E and therefore oxidative stress might not be an important factor in amyloid cystatin C cytotoxicity.

Tramiprosate is a drug designed for AD patients. It is thought to hinder the oligomerisation process. To test whether this drug could also prevent the oligomerisation process for the cystatin C amyloid, the drug was added to the same experimental setup.

When Tramiprosate alone was added to a culture of SMC, there is no visible toxic effect on the cell culture and when cystatin C amyloid was added to a SMC culture the same characteristic toxic effect were seen as former experiments had shown. When a mixture of both the Tramiposate drug and the amyloid cystatin C was added to a culture of SMC, a characteristic toxic effect was seen similar to the one seen when addition of only amyloid cystatin C was made.

When these results were quantified with the ATP-luciferase cell viability assay, the addition of the Tramiprosate did not affect the viability of the SMC culture. When the amyloid cystatin C was added to the culture, there was a drop in viability. When an addition of both Tramiprosate drug and the amyloid cystatin C was made, there was no reduction in the toxic effects when compared to the application of the cystatin C amyloid alone. Tramiprosate did therefore not show a significant sign as a therapeutic potential for HCHWA-I as reported for AD patients.

5.4. Cystatin C amyloid toxicity on PC12 cells

Cystatin C amyloid has been show to be toxic to SMC [174] but its effect on a neuronal model has not been researched. PC12 cells are a well-defined cell model for neuronal research *in vitro* and have been used extensively for testing Aβ peptide toxicity. This model can be easily differentiated with NGF to take on definite neuronal cell morphology. We observed sprouting of dendrites beginning on the first hour after applying NGF to the cell cultures. Six hours into the differentiation process, there were

branching of dendrites, which were already forming connection to their neighboring cells. After 2 days of differentiation, with NGF in the culture medium, the PC12 cells had formed an intricate net of dendrites connecting multiple cells. So it was decided that a differentiation of 2 days would be sufficient before testing the affects of the cystatin C amyloid.

To test whether the cystatin C amyloid might have toxic effect on a neurological model as well, the cells were differentiated and then different concentrations of cystatin C amyloid were added to the medium. With the addition of only 6.25 µM cystatin C, a visible cell clumping had begun. This clumping increased as the concentration of cystatin C amyloid increased.

For viability testing on these cells, the same experimental set up was used as for the SMC. Each concentration of amyloid cystatin C was tested by itself. The result showed a clear drop in cell viability indicating that cystatin C does have toxic affect on neuron-like cells, as well on SMC. This toxic affect does also seem to be in a dose-dependent manner, as was seen for the SMC toxicity. In immunohistochemical staining of tissue samples from HCHWA-I patients, a response of the neurons in the brain tissue has not been shown, whereas cells in the arterial walls are clearly affected [64, 174]. This might be because the brain tissue does not get introduced to the cystatin C amyloid. There are no amyloid plaques in HCHWA-I as it is in AD. Where the amyloid plaques are in AD patient´s brains, a clear degeneration of neuron tissue is seen around it [228]. These results indicate that the cystatin C amyloid might be toxic to neurons but the cells do not get exposed to the toxic form.

6. Appendix

Table 6.1 Statistical analysis for figure 4.8.

One Way Analysis of Variance

Normality Test: Passed (P = 0,125)

Equal Variance Test: Passed (P = 0,275)

Group Name	N	Missing	Mean	Std Dev	SEM
0,000	3	0	338,211	75,473	53,367
6,250	3	0	215,455	63,704	36,780
12,500	3	0	158,491	23,161	13,372
25,000	3	0	88,136	26,249	15,155
50,000	3	0	64,563	3,923	2,265
75,000	3	0	15,453	2,767	1,597
100,000	3	0	1,106	1,840	1,062

Source of Variation	DF	SS	MS	F	P
Between Groups	6	214033,797	35672,299	28,422	<0,001
Residual	13	16316,389	1255,107		
Total	19	230350,186			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0,000 vs. 100,000	337,105	10,424	<0,001	0,002	Yes
0,000 vs. 75,000	322,758	9,980	<0,001	0,003	Yes
0,000 vs. 50,000	273,648	8,461	<0,001	0,003	Yes
0,000 vs. 25,000	250,075	7,733	<0,001	0,003	Yes
6,250 vs. 100,000	214,349	7,410	<0,001	0,003	Yes
6,250 vs. 75,000	200,002	6,914	<0,001	0,003	Yes
0,000 vs. 12,500	179,720	5,557	<0,001	0,003	Yes
12,500 vs. 100,000	157,385	5,441	<0,001	0,004	Yes
6,250 vs. 50,000	150,892	5,216	<0,001	0,004	Yes
12,500 vs. 75,000	143,038	4,945	<0,001	0,004	Yes
6,250 vs. 25,000	127,319	4,401	<0,001	0,005	Yes
0,000 vs. 6,250	122,756	3,796	0,002	0,005	Yes
12,500 vs. 50,000	93,928	3,247	0,006	0,006	No
25,000 vs. 100,000	87,030	3,009	0,010	0,006	No
25,000 vs. 75,000	72,683	2,513	0,026	0,007	No
12,500 vs. 25,000	70,355	2,432	0,030	0,009	No
50,000 vs. 100,000	63,457	2,194	0,047	0,010	No
6,250 vs. 12,500	56,964	1,969	0,071	0,013	No
50,000 vs. 75,000	49,110	1,698	0,113	0,017	No
25,000 vs. 50,000	23,573	0,815	0,430	0,025	No
75,000 vs. 100,000	14,347	0,496	0,628	0,050	No

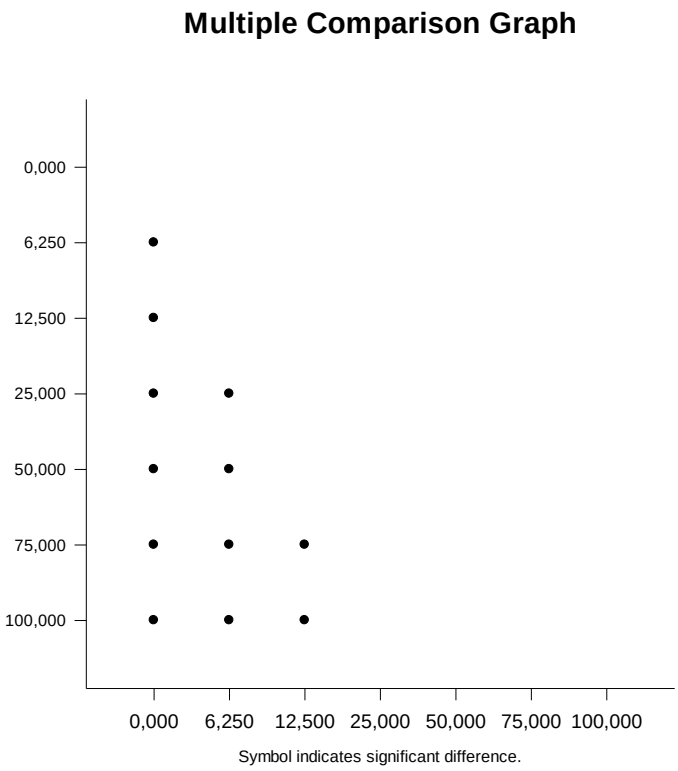

Figure 6.1 A multiple comparison graph for figure 4.8.

Table 6.2 Statistical analysis for figure 4.10.

One Way Analysis of Variance

Normality Test: Passed (P = 0,058)

Equal Variance Test: Passed (P = 0,854)

Group Name	N	Missing	Mean	Std Dev	SEM
control	7	0	100,000	7,859	2,971
cysC	7	0	56,828	13,975	5,282
Ab40	7	0	46,947	14,058	5,313
Ab42	7	0	75,829	14,067	5,317

Source of Variation	DF	SS	MS	F	P
Between Groups	3	11472,077	3824,026	23,440	<0,001
Residual	24	3915,342	163,139		
Total	27	15387,419			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
control vs. Ab40	53,053	7,771	<0,001	0,009	Yes
control vs. cysC	43,172	6,324	<0,001	0,010	Yes
Ab42 vs. Ab40	28,881	4,230	<0,001	0,013	Yes
control vs. Ab42	24,171	3,540	0,002	0,017	Yes
Ab42 vs. cysC	19,001	2,783	0,010	0,025	Yes
cysC vs. Ab40	9,880	1,447	0,161	0,050	No

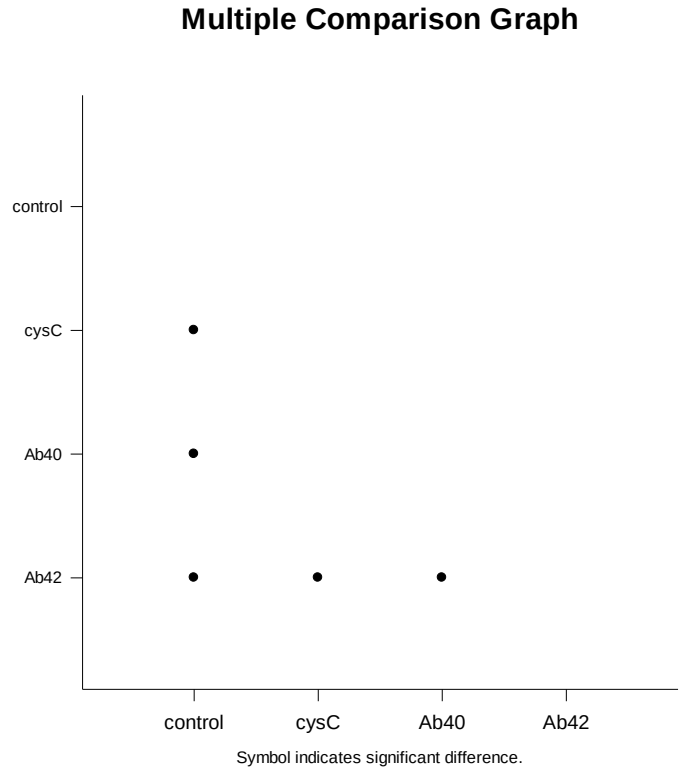

Figure 6.2 A multiple comparison graph for figure 4.10.

Table 6.3 Statistical analysis for figure 4.11.

One Way Analysis of Variance

Normality Test: Passed (P = 0,090)

Equal Variance Test: Passed (P = 0,434)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	5	0	100,000	9,339	4,177
CysC	5	0	77,279	16,000	7,155
Ab42	5	0	76,833	2,360	1,055
Ab40	5	0	57,867	10,647	4,762
CysC Ab42	5	0	63,965	7,511	3,359
CysC Ab40	5	0	50,092	6,625	2,963

Source of Variation	DF	SS	MS	F	P
Between Groups	5	7867,839	1573,568	16,786	<0,001
Residual	24	2249,786	93,741		
Total	29	10117,625			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
Control vs. CysC Ab40	49,908	8,150	<0,001	0,003	Yes
Control vs. Ab40	42,133	6,881	<0,001	0,004	Yes
Control vs. CysC Ab42	36,035	5,885	<0,001	0,004	Yes
CysC vs. CysC Ab40	27,187	4,440	<0,001	0,004	Yes
Ab42 vs. CysC Ab40	26,741	4,367	<0,001	0,005	Yes
Control vs. Ab42	23,167	3,783	<0,001	0,005	Yes
Control vs. CysC	22,721	3,711	0,001	0,006	Yes
CysC vs. Ab40	19,412	3,170	0,004	0,006	Yes
Ab42 vs. Ab40	18,966	3,097	0,005	0,007	Yes
CysC Ab42 vs. CysC Ab40	13,874	2,266	0,033	0,009	No
CysC vs. CysC Ab42	13,313	2,174	0,040	0,010	No
Ab42 vs. CysC Ab42	12,868	2,101	0,046	0,013	No
Ab40 vs. CysC Ab40	7,775	1,270	0,216	0,017	No
CysC Ab42 vs. Ab40	6,099	0,996	0,329	0,025	No
CysC vs. Ab42	0,445	0,0727	0,943	0,050	No

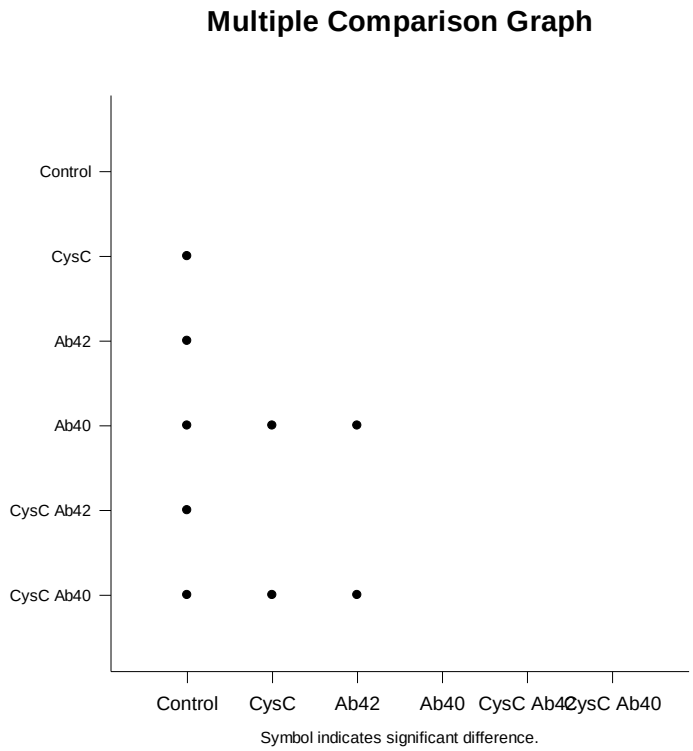

Figure 6.3 A multiple comparison graph for figure 4.11.

Table 6.4 Statistical analysis for figure 4.13.

One Way Analysis of Variance

Normality Test: Passed (P = 0,544)

Equal Variance Test: Passed (P = 0,381)

Group Name	N	Missing	Mean	Std Dev	SEM
control	5	0	100,000	6,340	2,835
HN	5	0	97,595	4,135	1,849
cysC	5	0	78,976	6,863	3,069
cysC+HN	5	0	80,395	9,657	4,319

Source of Variation	DF	SS	MS	F	P
Between Groups	3	1845,791	615,264	12,452	<0,001
Residual	16	790,582	49,411		
Total	19	2636,373			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 0,997

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
control vs. cysC	21,024	4,729	<0,001	0,009	Yes
control vs. cysC+HN	19,605	4,410	<0,001	0,010	Yes
HN vs. cysC	18,618	4,188	<0,001	0,013	Yes
HN vs. cysC+HN	17,200	3,869	0,001	0,017	Yes
control vs. HN	2,405	0,541	0,596	0,025	No
cysC+HN vs. cysC	1,418	0,319	0,754	0,050	No

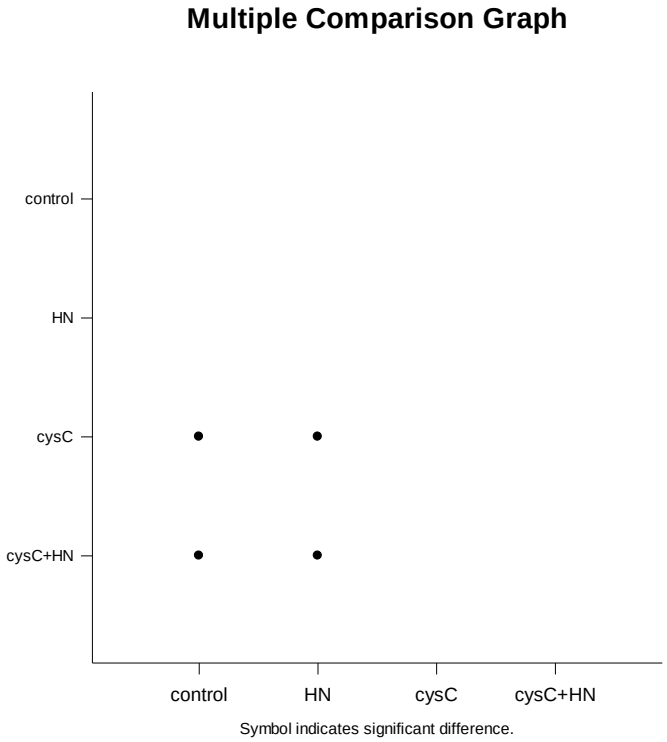

Figure 6.4 A multiple comparison graph for figure 4.13.

Table 6.5 Statistical analysis for figure 4.14.

One Way Analysis of Variance

Normality Test: Passed (P = 0,923)

Equal Variance Test: Passed (P = 0,063)

Group Name	N	Missing	Mean	Std Dev	SEM
control	6	0	100,000	7,960	3,249
HN	5	0	100,564	14,117	7,058
Ab40	6	0	51,492	4,378	1,787
Ab40+HN	6	0	60,967	6,973	2,847

Source of Variation	DF	SS	MS	F	P
Between Groups	3	10828,375	3609,458	51,829	<0,001
Residual	18	1253,558	69,642		
Total	21	12081,932			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
control vs. Ab40	48,508	10,068	<0,001	0,009	Yes
HN vs. Ab40	49,072	9,110	<0,001	0,010	Yes
control vs. Ab40+HN	39,033	8,101	<0,001	0,013	Yes
HN vs. Ab40+HN	39,597	7,351	<0,001	0,017	Yes
Ab40+HN vs. Ab40	9,475	1,967	0,065	0,025	No
HN vs. control	0,564	0,105	0,918	0,050	No

t-test

Normality Test: Passed (P = 0,592)

Equal Variance Test: Passed (P = 0,333)

Group Name	N	Missing	Mean	Std Dev	SEM
Ab40	6	0	51,492	4,378	1,787
Ab40+HN	6	0	60,967	6,973	2,847

Difference -9,475

t = -2,819 with 10 degrees of freedom. (P = 0,018)

95 percent confidence interval for difference of means: -16,964 to -1,986

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significant difference between the input groups (P = 0,018).

Power of performed test with alpha = 0,050: 0,665

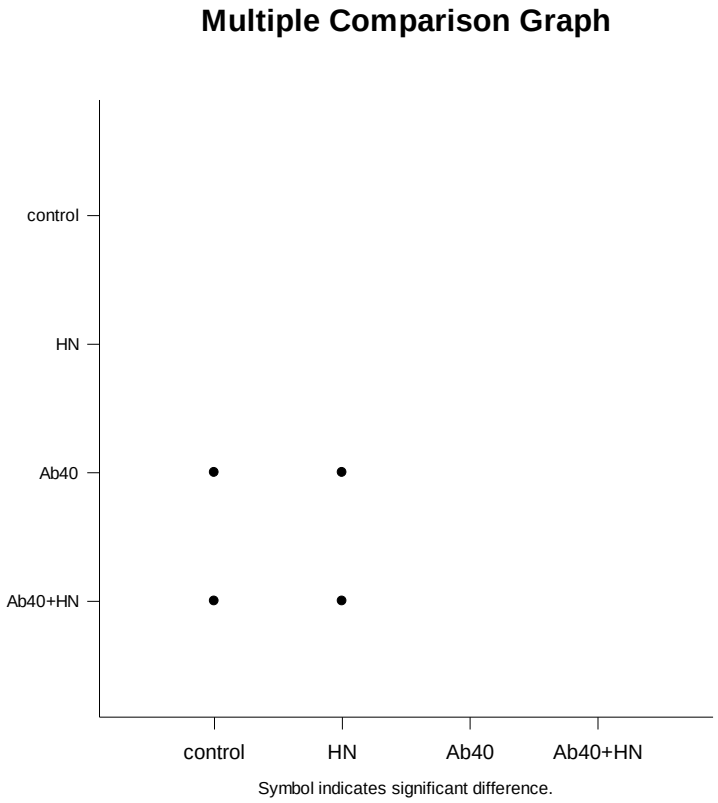

Figure 6.5 A multiple comparison graph for figure 4.14.

Table 6.6 Statistical analysis for figure 4.16.

One Way Analysis of Variance

Normality Test: Passed (P = 0,098)

Equal Variance Test: Passed (P = 0,293)

Group Name	N	Missing	Mean	Std Dev	SEM
Control	5	0	100,000	3,831	1,713
VitE 25 ug/mL	5	0	93,164	10,201	4,562
VitE 50 ug/mL	5	0	86,351	4,483	2,005
cysC	5	0	69,606	3,704	1,656
cysC + vitE 25 ug/mL	5	0	68,883	4,213	1,884
cysC + vitE 50 ug/mL	5	0	63,295	4,271	1,910

Source of Variation	DF	SS	MS	F	P
Between Groups	5	5620,171	1124,034	35,768	<0,001
Residual	24	754,213	31,426		
Total	29	6374,385			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**
Control vs. cysC + vitE 25	36,705	10,353	<0,001	0,003
Control vs. cysC + vitE 50	31,117	8,777	<0,001	0,004
Control vs. cysC	30,394	8,573	<0,001	0,004
VitE 25 ug/m vs. cysC + vitE 25	29,869	8,425	<0,001	0,004
VitE 25 ug/m vs. cysC + vitE 50	24,281	6,849	<0,001	0,005
VitE 25 ug/mL vs. cysC	23,558	6,644	<0,001	0,005
VitE 50 ug/m vs. cysC + vitE 25	23,056	6,503	<0,001	0,006
VitE 50 ug/m vs. cysC + vitE 50	17,468	4,927	<0,001	0,006
VitE 50 ug/mL vs. cysC	16,745	4,723	<0,001	0,007
Control vs. VitE 50 ug/mL	13,649	3,850	<0,001	0,009
Control vs. VitE 25 ug/mL	6,836	1,928	0,066	0,010
VitE 25 ug/m vs. VitE 50 ug/m	6,813	1,922	0,067	0,013
cysC vs. cysC + vitE 50 ug/mL	6,312	1,780	0,088	0,017
cysC + vitE 25 vs. cysC + vitE 50	5,588	1,576	0,128	0,025
cysC vs. cysC + vitE 25 ug/mL	0,723	0,204	0,840	0,050

Comparison	**Significant?**
Control vs. cysC + vitE 25	Yes
Control vs. cysC + vitE 50	Yes
Control vs. cysC	Yes
VitE 25 ug/m vs. cysC + vitE 25	Yes
VitE 25 ug/m vs. cysC + vitE 50	Yes
VitE 25 ug/mL vs. cysC	Yes
VitE 50 ug/m vs. cysC + vitE 25	Yes
VitE 50 ug/m vs. cysC + vitE 50	Yes
VitE 50 ug/mL vs. cysC	Yes
Control vs. VitE 50 ug/mL	Yes
Control vs. VitE 25 ug/mL	No
VitE 25 ug/m vs. VitE 50 ug/m	No
cysC vs. cysC + vitE 50 ug/mL	No
cysC + vitE vs. cysC + vitE	No
cysC vs. cysC + vitE 25 ug/mL	No

Multiple Comparison Graph

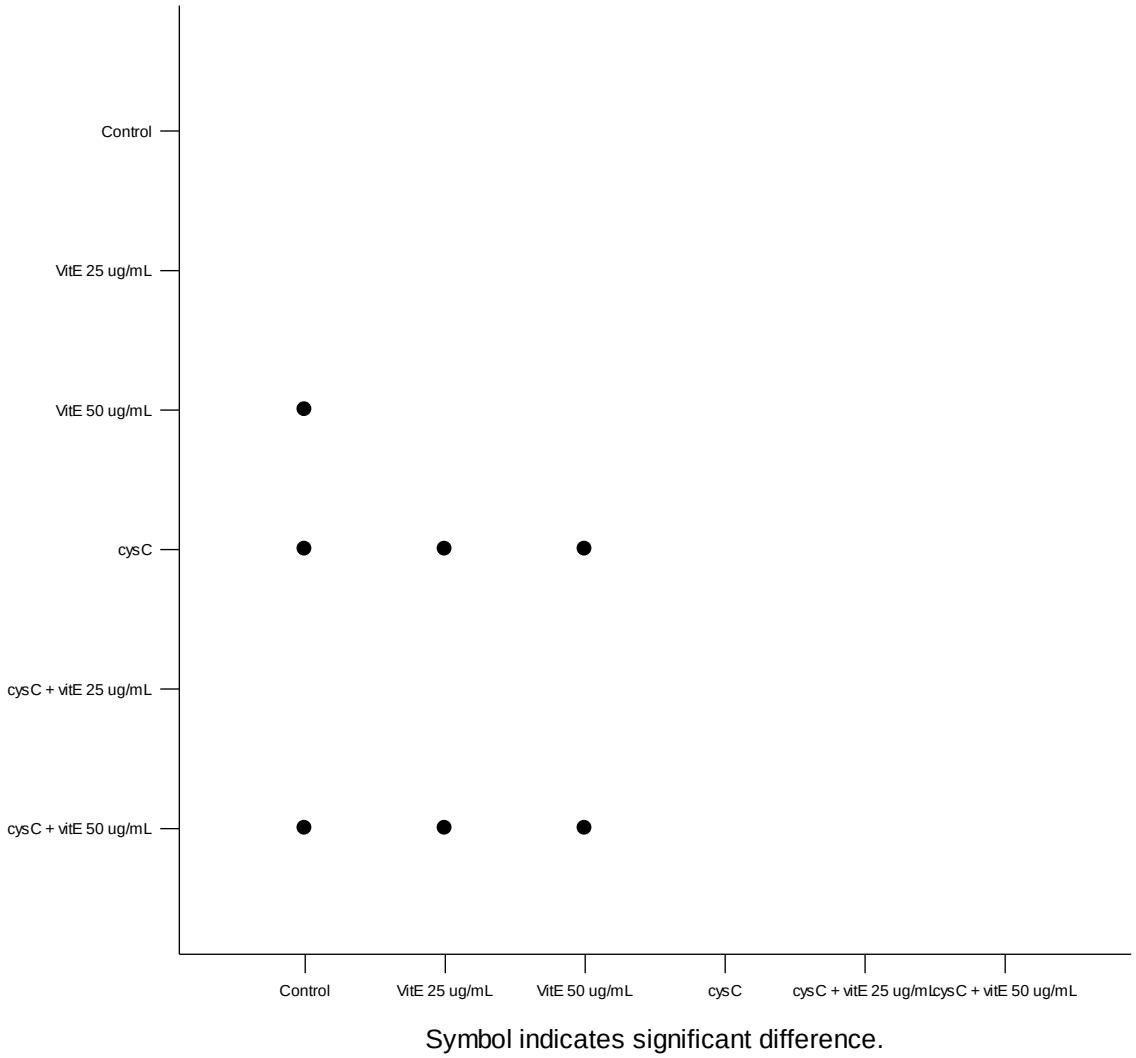

Figure 6.6 A multiple comparison graph for figure 4.16.

Table 6.7 Statistical analysis for figure 4.18.

One Way Analysis of Variance

Normality Test: Passed (P = 0,592)

Equal Variance Test: Passed (P = 0,473)

Group Name	N	Missing	Mean	Std Dev	SEM
control	4	0	100,000	4,160	2,080
3-APS	4	0	103,449	2,131	1,066
cysC	4	0	74,953	5,146	2,573
cysC+3-APS	4	0	76,531	5,406	2,703

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2729,046	909,682	46,918	<0,001
Residual	12	232,665	19,389		
Total	15	2961,711			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3-APS vs. cysC	28,495	9,152	<0,001	0,009	Yes
3-APS vs. cysC+3-APS	26,918	8,645	<0,001	0,010	Yes
control vs. cysC	25,047	8,044	<0,001	0,013	Yes
control vs. cysC+3-APS	23,469	7,538	<0,001	0,017	Yes
3-APS vs. control	3,449	1,108	0,290	0,025	No
cysC+3-APS vs. cysC	1,578	0,507	0,622	0,050	No

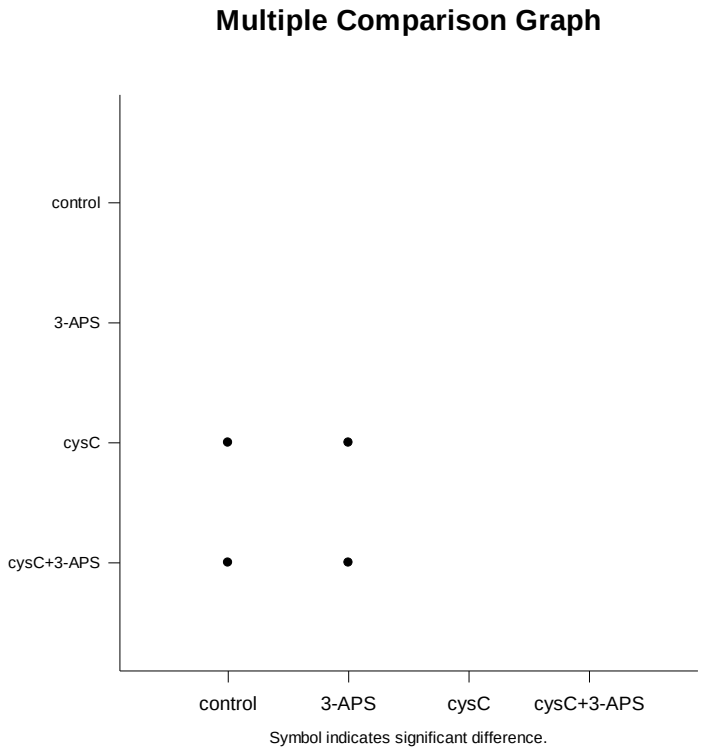

Figure 6.7 A multiple comparison graph for figure 4.18.

Table 6.8 Statistical analysis for figure 4.21.

One Way Analysis of Variance

Normality Test: Passed (P = 0,086)

Equal Variance Test: Passed (P = 0,838)

Group Name	N	Missing	Mean	Std Dev	SEM
0 uM	4	0	33,412	2,294	1,147
12 uM	5	0	10,833	0,735	0,329
50 uM	5	0	2,317	1,150	0,514
75 uM	5	0	1,180	1,022	0,457

Source of Variation	DF	SS	MS	F	P
Between Groups	3	2867,743	955,914	522,929	<0,001
Residual	15	27,420	1,828		
Total	18	2895,163			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0,001).

Power of performed test with alpha = 0,050: 1,000

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0,05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
0 uM vs. 75 uM	32,232	35,538	<0,001	0,009	Yes
0 uM vs. 50 uM	31,095	34,284	<0,001	0,010	Yes
0 uM vs. 12 uM	22,580	24,896	<0,001	0,013	Yes
12 uM vs. 75 uM	9,652	11,288	<0,001	0,017	Yes
12 uM vs. 50 uM	8,515	9,958	<0,001	0,025	Yes
50 uM vs. 75 uM	1,137	1,330	0,203	0,050	No

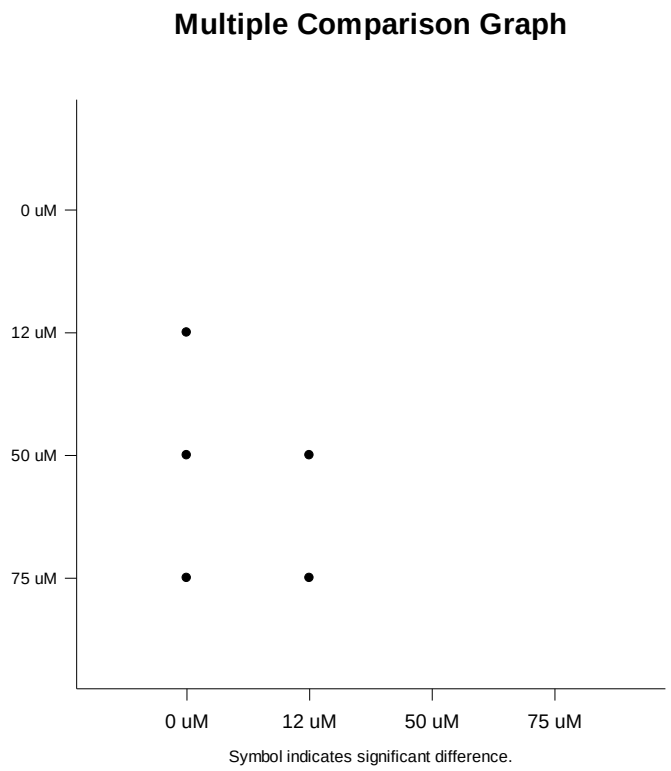

Figure 6.8 A multiple comparison graph for figure 4.21.

REFERENCES

1. Merlini, G. and V. Bellotti, *Molecular mechanisms of amyloidosis.* N Engl J Med, 2003. **349**(6): p. 583-96.
2. Revesz, T., et al., *Cerebral amyloid angiopathies: a pathologic, biochemical, and genetic view.* J Neuropathol Exp Neurol, 2003. **62**(9): p. 885-98.
3. Stefani, M. and C.M. Dobson, *Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution.* J Mol Med (Berl), 2003. **81**(11): p. 678-99.
4. Thanvi, B. and T. Robinson, *Sporadic cerebral amyloid angiopathy--an important cause of cerebral haemorrhage in older people.* Age Ageing, 2006. **35**(6): p. 565-71.
5. Prusiner, S.B., et al., *Prion protein biology.* Cell, 1998. **93**(3): p. 337-48.
6. Liberski, P.P., *The transmissible brain amyloidoses: a comparison with the non transmissible brain amyloidoses of Alzheimer type.* Acta Neurobiol Exp (Wars), 1993. **53**(1): p. 337-49.
7. Bardin, T., et al., *Hemodialysis-associated amyloidosis and beta-2 microglobulin. Clinical and immunohistochemical study.* Am J Med, 1987. **83**(3): p. 419-24.
8. Yan, S.D., et al., *Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis.* Nat Med, 2000. **6**(6): p. 643-51.
9. Hardy, J. and D.J. Selkoe, *The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics.* Science, 2002. **297**(5580): p. 353-6.
10. Dobson, C.M., *The structural basis of protein folding and its links with human disease.* Philos Trans R Soc Lond B Biol Sci, 2001. **356**(1406): p. 133-45.
11. Puchtler, H. and F. Sweat, *Congo red as a stain for fluorescence microscopy of amyloid.* J Histochem Cytochem, 1965. **13**(8): p. 693-4.
12. Xing, Y. and K. Higuchi, *Amyloid fibril proteins.* Mech Ageing Dev, 2002. **123**(12): p. 1625-36.
13. Glenner, G.G. and C.W. Wong, *Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein.* Biochem Biophys Res Commun, 1984. **122**(3): p. 1131-5.
14. Cecchi, C., et al., *Insights into the molecular basis of the differing susceptibility of varying cell types to the toxicity of amyloid aggregates.* J Cell Sci, 2005. **118**(Pt 15): p. 3459-70.
15. Tjernberg, L., et al., *Charge attraction and beta propensity are necessary for amyloid fibril formation from tetrapeptides.* J Biol Chem, 2002. **277**(45): p. 43243-6.
16. Ghiso, J., O. Jensson, and B. Frangione, *Amyloid fibrils in hereditary cerebral hemorrhage with amyloidosis of Icelandic type is a variant of gamma-trace basic protein (cystatin C).* Proc Natl Acad Sci U S A, 1986. **83**(9): p. 2974-8.
17. Ghiso, J. and B. Frangione, *Amyloidosis and Alzheimer's disease.* Adv Drug Deliv Rev, 2002. **54**(12): p. 1539-51.
18. Vinters, H.V., *Cerebral amyloid angiopathy. A critical review.* Stroke, 1987. **18**(2): p. 311-24.
19. Pardridge, W.M., *Molecular biology of the blood-brain barrier.* Mol Biotechnol, 2005. **30**(1): p. 57-70.
20. Maia, L.F., I.R. Mackenzie, and H.H. Feldman, *Clinical phenotypes of Cerebral Amyloid Angiopathy.* J Neurol Sci, 2007. **257**(1-2): p. 23-30.
21. Vonsattel, J.P., et al., *Cerebral amyloid angiopathy without and with cerebral hemorrhages: a comparative histological study.* Ann Neurol, 1991. **30**(5): p. 637-49.
22. Mandybur, T.I., *Cerebral amyloid angiopathy: the vascular pathology and complications.* J Neuropathol Exp Neurol, 1986. **45**(1): p. 79-90.
23. Okoye, M.I. and I. Watanabe, *Ultrastructural features of cerebral amyloid angiopathy.* Hum Pathol, 1982. **13**(12): p. 1127-32.

24. Revesz, T., et al., *Sporadic and familial cerebral amyloid angiopathies.* Brain Pathol, 2002. **12**(3): p. 343-57.
25. Zipfel, G.J., et al., *Cerebral amyloid angiopathy: progressive disruption of the neurovascular unit.* Stroke, 2009. **40**(3 Suppl): p. S16-9.
26. Torack, R.M., *Congophilic angiopathy complicated by surgery and massive hemorrhage. A light and electron microscopic study.* Am J Pathol, 1975. **81**(2): p. 349-66.
27. McGrath, M.E., *The lysosomal cysteine proteases.* Annu Rev Biophys Biomol Struct, 1999. **28**: p. 181-204.
28. Bjorck, L., A. Grubb, and L. Kjellen, *Cystatin C, a human proteinase inhibitor, blocks replication of herpes simplex virus.* J Virol, 1990. **64**(2): p. 941-3.
29. Bjorck, L., et al., *Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor.* Nature, 1989. **337**(6205): p. 385-6.
30. Ochieng, J. and G. Chaudhuri, *Cystatin superfamily.* J Health Care Poor Underserved. **21**(1 Suppl): p. 51-70.
31. Abrahamson, M., et al., *Structure and expression of the human cystatin C gene.* Biochem J, 1990. **268**(2): p. 287-94.
32. Bobek, L.A. and M.J. Levine, *Cystatins--inhibitors of cysteine proteinases.* Crit Rev Oral Biol Med, 1992. **3**(4): p. 307-32.
33. Saitoh, E., et al., *The human cystatin C gene (CST3) is a member of the cystatin gene family which is localized on chromosome 20.* Biochem Biophys Res Commun, 1989. **162**(3): p. 1324-31.
34. Turk, V. and W. Bode, *The cystatins: protein inhibitors of cysteine proteinases.* FEBS Lett, 1991. **285**(2): p. 213-9.
35. Mussap, M. and M. Plebani, *Biochemistry and clinical role of human cystatin C.* Crit Rev Clin Lab Sci, 2004. **41**(5-6): p. 467-550.
36. Abrahamson, M., et al., *Human cystatin C. role of the N-terminal segment in the inhibition of human cysteine proteinases and in its inactivation by leucocyte elastase.* Biochem J, 1991. **273 (Pt 3)**: p. 621-6.
37. Benedikz, E., H. Blondal, and G. Gudmundsson, *Skin deposits in hereditary cystatin C amyloidosis.* Virchows Arch A Pathol Anat Histopathol, 1990. **417**(4): p. 325-31.
38. Palm, D.E., et al., *Cystatin C, a protease inhibitor, in degenerating rat hippocampal neurons following transient forebrain ischemia.* Brain Res, 1995. **691**(1-2): p. 1-8.
39. Kopitar-Jerala, N., *The role of cystatins in cells of the immune system.* FEBS Lett, 2006. **580**(27): p. 6295-301.
40. Palsdottir, A., A.O. Snorradottir, and L. Thorsteinsson, *Hereditary cystatin C amyloid angiopathy: genetic, clinical, and pathological aspects.* Brain Pathol, 2006. **16**(1): p. 55-9.
41. Sveinbjornsdottir, S., et al., *Progressive dementia and leucoencephalopathy as the initial presentation of late onset hereditary cystatin-C amyloidosis. Clinicopathological presentation of two cases.* J Neurol Sci, 1996. **140**(1-2): p. 101-8.
42. Gudmundsson, G., et al., *Hereditary cerebral haemorrhage with amyloidosis.* Brain, 1972. **95**(2): p. 387-404.
43. Jensson, O., et al., *Hereditary central nervous system γ-trace amyloid angiopathy and stroke in Icelandic families*, in *AMYLOIDOSIS*, G.G. Glenner, et al., Editors. 1986, Plenum Publishing Corporation. p. 789-801.
44. Cohen, D.H., et al., *Amyloid fibril in hereditary cerebral hemorrhage with amyloidosis (HCHWA) is related to the gastroentero-pancreatic neuroendocrine protein, gamma trace.* J Exp Med, 1983. **158**(2): p. 623-8.
45. Abrahamson, M., et al., *The human cystatin C gene (CST3), mutated in hereditary cystatin C amyloid angiopathy, is located on chromosome 20.* Hum Genet, 1989. **82**(3): p. 223-6.
46. Levy, E., et al., *Stroke in Icelandic patients with hereditary amyloid angiopathy is related to a mutation in the cystatin C gene, an inhibitor of cysteine proteases.* J Exp Med, 1989. **169**(5): p. 1771-8.

47. Palsdottir, A., et al., *Mutation in the cystatin C gene causes hereditary brain hemorrhage.* Prog Clin Biol Res, 1989. **317**: p. 241-6.
48. Grubb, A. and H. Lofberg, *Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis.* Proc Natl Acad Sci U S A, 1982. **79**(9): p. 3024-7.
49. Asgeirsson, B., et al., *Hereditary cystatin C amyloid angiopathy: monitoring the presence of the Leu-68-->Gln cystatin C variant in cerebrospinal fluids and monocyte cultures by MS.* Biochem J, 1998. **329 (Pt 3)**: p. 497-503.
50. Wei, L., et al., *Instability of the amyloidogenic cystatin C variant of hereditary cerebral hemorrhage with amyloidosis, Icelandic type.* J Biol Chem, 1998. **273**(19): p. 11806-14.
51. Lofberg, H., et al., *Immunohistochemical characterization of the amyloid deposits and quantitation of pertinent cerebrospinal fluid proteins in hereditary cerebral hemorrhage with amyloidosis.* Stroke, 1987. **18**(2): p. 431-40.
52. Olafsson, I., et al., *The amino terminal portion of cerebrospinal fluid cystatin C in hereditary cystatin C amyloid angiopathy is not truncated: direct sequence analysis from agarose gel electropherograms.* Scand J Clin Lab Invest, 1990. **50**(1): p. 85-93.
53. Hall, A., et al., *Importance of the evolutionarily conserved glycine residue in the N-terminal region of human cystatin C (Gly-11) for cysteine endopeptidase inhibition.* Biochem J, 1993. **291 (Pt 1)**: p. 123-9.
54. Abrahamson, M., et al., *Identification of the probable inhibitory reactive sites of the cysteine proteinase inhibitors human cystatin C and chicken cystatin.* J Biol Chem, 1987. **262**(20): p. 9688-94.
55. Gerhartz, B. and M. Abrahamson, *Physico-chemical properties of the N-terminally truncated L68Q cystatin C found in amyloid deposits of brain haemorrhage patients.* Biol Chem, 2002. **383**(2): p. 301-5.
56. Gerhartz, B., I. Ekiel, and M. Abrahamson, *Two stable unfolding intermediates of the disease-causing L68Q variant of human cystatin C.* Biochemistry, 1998. **37**(49): p. 17309-17.
57. Abrahamson, M. and A. Grubb, *Increased body temperature accelerates aggregation of the Leu-68-->Gln mutant cystatin C, the amyloid-forming protein in hereditary cystatin C amyloid angiopathy.* Proc Natl Acad Sci U S A, 1994. **91**(4): p. 1416-20.
58. Calero, M., et al., *Distinct properties of wild-type and the amyloidogenic human cystatin C variant of hereditary cerebral hemorrhage with amyloidosis, Icelandic type.* J Neurochem, 2001. **77**(2): p. 628-37.
59. Bjarnadottir, M., et al., *Intracellular accumulation of the amyloidogenic L68Q variant of human cystatin C in NIH/3T3 cells.* Mol Pathol, 1998. **51**(6): p. 317-26.
60. Ekiel, I., et al., *NMR structural studies of human cystatin C dimers and monomers.* J Mol Biol, 1997. **271**(2): p. 266-77.
61. Janowski, R., et al., *Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping.* Nat Struct Biol, 2001. **8**(4): p. 316-20.
62. Lofberg, H. and A.O. Grubb, *Quantitation of gamma-trace in human biological fluids: indications for production in the central nervous system.* Scand J Clin Lab Invest, 1979. **39**(7): p. 619-26.
63. Olafsson, I., L. Thorsteinsson, and O. Jensson, *The molecular pathology of hereditary cystatin C amyloid angiopathy causing brain hemorrhage.* Brain Pathol, 1996. **6**(2): p. 121-6.
64. Blöndal, H., *Ultrastructural aspects of hereditary central nervous system amyloid angiopathy.* Acta Nerol Scand, 1986. **73**: p. 319.
65. Olafsson, I. and A. Grubb, *Hereditary cystatin C amyloid angiopathy.* Amyloid, 2000. **7**(1): p. 70-9.
66. Wang, Z.Z., et al., *Microvascular degeneration in hereditary cystatin C amyloid angiopathy of the brain.* APMIS, 1997. **105**(1): p. 41-7.
67. Bjarnadottir, M., et al., *The cerebral hemorrhage-producing cystatin C variant (L68Q) in extracellular fluids.* Amyloid, 2001. **8**(1): p. 1-10.

68. McKhann, G.M., et al., *The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease.* Alzheimers Dement. **7**(3): p. 263-9.
69. Holtzman, D.M., J.C. Morris, and A.M. Goate, *Alzheimer's disease: the challenge of the second century.* Sci Transl Med. **3**(77): p. 77sr1.
70. Terry, R.D., et al., *Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment.* Ann Neurol, 1991. **30**(4): p. 572-80.
71. Grundke-Iqbal, I., et al., *Microtubule-associated protein tau. A component of Alzheimer paired helical filaments.* J Biol Chem, 1986. **261**(13): p. 6084-9.
72. Kosik, K.S., C.L. Joachim, and D.J. Selkoe, *Microtubule-associated protein tau (tau) is a major antigenic component of paired helical filaments in Alzheimer disease.* Proc Natl Acad Sci U S A, 1986. **83**(11): p. 4044-8.
73. Glenner, G.G. and C.W. Wong, *Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein.* Biochem Biophys Res Commun, 1984. **120**(3): p. 885-90.
74. Masters, C.L., et al., *Amyloid plaque core protein in Alzheimer disease and Down syndrome.* Proc Natl Acad Sci U S A, 1985. **82**(12): p. 4245-9.
75. Price, J.L., et al., *Neuropathology of nondemented aging: presumptive evidence for preclinical Alzheimer disease.* Neurobiol Aging, 2009. **30**(7): p. 1026-36.
76. Perrin, R.J., A.M. Fagan, and D.M. Holtzman, *Multimodal techniques for diagnosis and prognosis of Alzheimer's disease.* Nature, 2009. **461**(7266): p. 916-22.
77. Neve, R.L., D.L. McPhie, and Y. Chen, *Alzheimer's disease: a dysfunction of the amyloid precursor protein(1).* Brain Res, 2000. **886**(1-2): p. 54-66.
78. Lamb, B.T., et al., *Introduction and expression of the 400 kilobase amyloid precursor protein gene in transgenic mice [corrected].* Nat Genet, 1993. **5**(1): p. 22-30.
79. Konig, G., et al., *Identification and differential expression of a novel alternative splice isoform of the beta A4 amyloid precursor protein (APP) mRNA in leukocytes and brain microglial cells.* J Biol Chem, 1992. **267**(15): p. 10804-9.
80. Lai, F. and R.S. Williams, *A prospective study of Alzheimer disease in Down syndrome.* Arch Neurol, 1989. **46**(8): p. 849-53.
81. Qiu, W.Q., et al., *Cell-surface beta-amyloid precursor protein stimulates neurite outgrowth of hippocampal neurons in an isoform-dependent manner.* J Neurosci, 1995. **15**(3 Pt 2): p. 2157-67.
82. Smith, R.P., D.A. Higuchi, and G.J. Broze, Jr., *Platelet coagulation factor XIa-inhibitor, a form of Alzheimer amyloid precursor protein.* Science, 1990. **248**(4959): p. 1126-8.
83. Schubert, D., et al., *The regulation of amyloid beta protein precursor secretion and its modulatory role in cell adhesion.* Neuron, 1989. **3**(6): p. 689-94.
84. Nikolaev, A., et al., *APP binds DR6 to trigger axon pruning and neuron death via distinct caspases.* Nature, 2009. **457**(7232): p. 981-9.
85. Zheng, P., et al., *PAT1, a microtubule-interacting protein, recognizes the basolateral sorting signal of amyloid precursor protein.* Proc Natl Acad Sci U S A, 1998. **95**(25): p. 14745-50.
86. Saitoh, T., et al., *Secreted form of amyloid beta protein precursor is involved in the growth regulation of fibroblasts.* Cell, 1989. **58**(4): p. 615-22.
87. Ring, S., et al., *The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral, and electrophysiological abnormalities of APP-deficient mice.* J Neurosci, 2007. **27**(29): p. 7817-26.
88. Seubert, P., et al., *Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids.* Nature, 1992. **359**(6393): p. 325-7.
89. Cummings, B.J., et al., *Beta-amyloid deposition and other measures of neuropathology predict cognitive status in Alzheimer's disease.* Neurobiol Aging, 1996. **17**(6): p. 921-33.

90. Kamenetz, F., et al., *APP processing and synaptic function.* Neuron, 2003. **37**(6): p. 925-37.
91. Pike, C.J., M.J. Overman, and C.W. Cotman, *Amino-terminal deletions enhance aggregation of beta-amyloid peptides in vitro.* J Biol Chem, 1995. **270**(41): p. 23895-8.
92. Shoji, M., et al., *Production of the Alzheimer amyloid beta protein by normal proteolytic processing.* Science, 1992. **258**(5079): p. 126-9.
93. Wisniewski, T. and B. Frangione, *Apolipoprotein E: a pathological chaperone protein in patients with cerebral and systemic amyloid.* Neurosci Lett, 1992. **135**(2): p. 235-8.
94. Ghiso, J., et al., *The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex.* Biochem J, 1993. **293 (Pt 1)**: p. 27-30.
95. Suzuki, N., et al., *An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants.* Science, 1994. **264**(5163): p. 1336-40.
96. Iwatsubo, T., et al., *Visualization of A beta 42(43) and A beta 40 in senile plaques with end-specific A beta monoclonals: evidence that an initially deposited species is A beta 42(43).* Neuron, 1994. **13**(1): p. 45-53.
97. Mandelkow, E.M., et al., *Tau domains, phosphorylation, and interactions with microtubules.* Neurobiol Aging, 1995. **16**(3): p. 355-62; discussion 362-3.
98. Grundke-Iqbal, I., et al., *Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology.* Proc Natl Acad Sci U S A, 1986. **83**(13): p. 4913-7.
99. Morishima-Kawashima, M., et al., *Hyperphosphorylation of tau in PHF.* Neurobiol Aging, 1995. **16**(3): p. 365-71; discussion 371-80.
100. Silverman, W., et al., *Frequency of stages of Alzheimer-related lesions in different age categories.* Neurobiol Aging, 1997. **18**(4): p. 377-9; discussion 389-92.
101. Takashima, A., et al., *Tau protein kinase I is essential for amyloid beta-protein-induced neurotoxicity.* Proc Natl Acad Sci U S A, 1993. **90**(16): p. 7789-93.
102. Frost, B. and M.I. Diamond, *Prion-like mechanisms in neurodegenerative diseases.* Nat Rev Neurosci. **11**(3): p. 155-9.
103. Meyer-Luehmann, M., et al., *Exogenous induction of cerebral beta-amyloidogenesis is governed by agent and host.* Science, 2006. **313**(5794): p. 1781-4.
104. Selkoe, D.J., *Alzheimer's disease: genes, proteins, and therapy.* Physiol Rev, 2001. **81**(2): p. 741-66.
105. Xia, W., et al., *Interaction between amyloid precursor protein and presenilins in mammalian cells: implications for the pathogenesis of Alzheimer disease.* Proc Natl Acad Sci U S A, 1997. **94**(15): p. 8208-13.
106. Hardy, J., *The Alzheimer family of diseases: many etiologies, one pathogenesis?* Proc Natl Acad Sci U S A, 1997. **94**(6): p. 2095-7.
107. Irizarry, M.C., et al., *Abeta deposition is associated with neuropil changes, but not with overt neuronal loss in the human amyloid precursor protein V717F (PDAPP) transgenic mouse.* J Neurosci, 1997. **17**(18): p. 7053-9.
108. Benilova, I., E. Karran, and B. De Strooper, *The toxic Abeta oligomer and Alzheimer's disease: an emperor in need of clothes.* Nat Neurosci, 2012. **15**(3): p. 349-57.
109. Wisniewski, T., J. Ghiso, and B. Frangione, *Biology of A beta amyloid in Alzheimer's disease.* Neurobiol Dis, 1997. **4**(5): p. 313-28.
110. Pike, C.J., et al., *In vitro aging of beta-amyloid protein causes peptide aggregation and neurotoxicity.* Brain Res, 1991. **563**(1-2): p. 311-4.
111. Walsh, D.M., et al., *Amyloid beta-protein fibrillogenesis. Detection of a protofibrillar intermediate.* J Biol Chem, 1997. **272**(35): p. 22364-72.
112. McLean, C.A., et al., *Soluble pool of Abeta amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease.* Ann Neurol, 1999. **46**(6): p. 860-6.

113. Mc Donald, J.M., et al., *The presence of sodium dodecyl sulphate-stable Abeta dimers is strongly associated with Alzheimer-type dementia.* Brain. **133**(Pt 5): p. 1328-41.
114. Haass, C. and D.J. Selkoe, *Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide.* Nat Rev Mol Cell Biol, 2007. **8**(2): p. 101-12.
115. Harper, J.D., et al., *Observation of metastable Abeta amyloid protofibrils by atomic force microscopy.* Chem Biol, 1997. **4**(2): p. 119-25.
116. Klein, W.L., G.A. Krafft, and C.E. Finch, *Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum?* Trends Neurosci, 2001. **24**(4): p. 219-24.
117. Ono, K., M.M. Condron, and D.B. Teplow, *Structure-neurotoxicity relationships of amyloid beta-protein oligomers.* Proc Natl Acad Sci U S A, 2009. **106**(35): p. 14745-50.
118. Lue, L.F., et al., *Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease.* Am J Pathol, 1999. **155**(3): p. 853-62.
119. Westerman, M.A., et al., *The relationship between Abeta and memory in the Tg2576 mouse model of Alzheimer's disease.* J Neurosci, 2002. **22**(5): p. 1858-67.
120. Verdier, Y. and B. Penke, *Binding sites of amyloid beta-peptide in cell plasma membrane and implications for Alzheimer's disease.* Curr Protein Pept Sci, 2004. **5**(1): p. 19-31.
121. Snyder, E.M., et al., *Regulation of NMDA receptor trafficking by amyloid-beta.* Nat Neurosci, 2005. **8**(8): p. 1051-8.
122. Yankner, B.A., L.K. Duffy, and D.A. Kirschner, *Neurotrophic and neurotoxic effects of amyloid beta protein: reversal by tachykinin neuropeptides.* Science, 1990. **250**(4978): p. 279-82.
123. Boland, K., et al., *The serpin-enzyme complex receptor recognizes soluble, nontoxic amyloid-beta peptide but not aggregated, cytotoxic amyloid-beta peptide.* J Biol Chem, 1996. **271**(30): p. 18032-44.
124. Yan, S.D., et al., *RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease.* Nature, 1996. **382**(6593): p. 685-91.
125. Mattson, M.P., et al., *beta-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity.* J Neurosci, 1992. **12**(2): p. 376-89.
126. Ye, C., et al., *Amyloid-beta proteins activate Ca(2+)-permeable channels through calcium-sensing receptors.* J Neurosci Res, 1997. **47**(5): p. 547-54.
127. Whitson, J.S. and S.H. Appel, *Neurotoxicity of A beta amyloid protein in vitro is not altered by calcium channel blockade.* Neurobiol Aging, 1995. **16**(1): p. 5-10.
128. Arispe, N., E. Rojas, and H.B. Pollard, *Alzheimer disease amyloid beta protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum.* Proc Natl Acad Sci U S A, 1993. **90**(2): p. 567-71.
129. Lin, H., R. Bhatia, and R. Lal, *Amyloid beta protein forms ion channels: implications for Alzheimer's disease pathophysiology.* FASEB J, 2001. **15**(13): p. 2433-44.
130. Arispe, N., J.C. Diaz, and O. Simakova, *Abeta ion channels. Prospects for treating Alzheimer's disease with Abeta channel blockers.* Biochim Biophys Acta, 2007. **1768**(8): p. 1952-65.
131. Allen, D.D., et al., *Beta-amyloid induced increase in choline flux across PC12 cell membranes.* Neurosci Lett, 1997. **234**(1): p. 71-3.
132. Thomas, T., et al., *Cerebrovascular endothelial dysfunction mediated by beta-amyloid.* Neuroreport, 1997. **8**(6): p. 1387-91.
133. Demuro, A., et al., *Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers.* J Biol Chem, 2005. **280**(17): p. 17294-300.
134. Arispe, N., H.B. Pollard, and E. Rojas, *Giant multilevel cation channels formed by Alzheimer disease amyloid beta-protein [A beta P-(1-40)] in bilayer membranes.* Proc Natl Acad Sci U S A, 1993. **90**(22): p. 10573-7.

135. Kagan, B.L., et al., *The channel hypothesis of Alzheimer's disease: current status.* Peptides, 2002. **23**(7): p. 1311-5.
136. Simakova, O. and N.J. Arispe, *Early and late cytotoxic effects of external application of the Alzheimer's Abeta result from the initial formation and function of Abeta ion channels.* Biochemistry, 2006. **45**(18): p. 5907-15.
137. Moreira, P.I., et al., *Amyloid beta-peptide promotes permeability transition pore in brain mitochondria.* Biosci Rep, 2001. **21**(6): p. 789-800.
138. Harman, D., *The aging process.* Proc Natl Acad Sci U S A, 1981. **78**(11): p. 7124-8.
139. Sayre, L.M., G. Perry, and M.A. Smith, *Oxidative stress and neurotoxicity.* Chem Res Toxicol, 2008. **21**(1): p. 172-88.
140. Huang, X., et al., *The A beta peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction.* Biochemistry, 1999. **38**(24): p. 7609-16.
141. Kim, H.Y., *Novel metabolism of docosahexaenoic acid in neural cells.* J Biol Chem, 2007. **282**(26): p. 18661-5.
142. Peng, Z.F., et al., *Deciphering the mechanism of HNE-induced apoptosis in cultured murine cortical neurons: transcriptional responses and cellular pathways.* Neuropharmacology, 2007. **53**(5): p. 687-98.
143. Varadarajan, S., et al., *Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity.* J Struct Biol, 2000. **130**(2-3): p. 184-208.
144. Nunomura, A., et al., *Oxidative damage is the earliest event in Alzheimer disease.* J Neuropathol Exp Neurol, 2001. **60**(8): p. 759-67.
145. Huang, X., et al., *Cu(II) potentiation of alzheimer abeta neurotoxicity. Correlation with cell-free hydrogen peroxide production and metal reduction.* J Biol Chem, 1999. **274**(52): p. 37111-6.
146. Kuperstein, I., et al., *Neurotoxicity of Alzheimer's disease Abeta peptides is induced by small changes in the Abeta42 to Abeta40 ratio.* EMBO J, 2010. **29**(19): p. 3408-20.
147. Haass, C. and D.J. Selkoe, *Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide.* Cell, 1993. **75**(6): p. 1039-42.
148. Scheuner, D., et al., *Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease.* Nat Med, 1996. **2**(8): p. 864-70.
149. Kim, J., et al., *Abeta40 inhibits amyloid deposition in vivo.* J Neurosci, 2007. **27**(3): p. 627-33.
150. De Strooper, B., *Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease.* EMBO Rep, 2007. **8**(2): p. 141-6.
151. Frost, D., et al., *Co-incorporation of A beta 40 and A beta 42 to form mixed pre-fibrillar aggregates.* Eur J Biochem, 2003. **270**(4): p. 654-63.
152. Snyder, S.W., et al., *Amyloid-beta aggregation: selective inhibition of aggregation in mixtures of amyloid with different chain lengths.* Biophys J, 1994. **67**(3): p. 1216-28.
153. Maruyama, K., et al., *Immunohistochemical characterization of cerebrovascular amyloid in 46 autopsied cases using antibodies to beta protein and cystatin C.* Stroke, 1990. **21**(3): p. 397-403.
154. Vinters, H.V., et al., *Immunoreactive A4 and gamma-trace peptide colocalization in amyloidotic arteriolar lesions in brains of patients with Alzheimer's disease.* Am J Pathol, 1990. **137**(2): p. 233-40.
155. Levy, E., et al., *Codeposition of cystatin C with amyloid-beta protein in the brain of Alzheimer disease patients.* J Neuropathol Exp Neurol, 2001. **60**(1): p. 94-104.
156. Haan, J., et al., *Co-localization of beta/A4 and cystatin C in cortical blood vessels in Dutch, but not in Icelandic hereditary cerebral hemorrhage with amyloidosis.* Acta Neurol Scand, 1994. **89**(5): p. 367-71.
157. Maruyama, K., et al., *Characterization of amyloid fibril protein from a case of cerebral amyloid angiopathy showing immunohistochemical reactivity for both beta protein and cystatin C.* Neurosci Lett, 1992. **144**(1-2): p. 38-42.

158. Mi, W., et al., *Cystatin C inhibits amyloid-beta deposition in Alzheimer's disease mouse models.* Nat Genet, 2007. **39**(12): p. 1440-2.
159. Sastre, M., et al., *Binding of cystatin C to Alzheimer's amyloid beta inhibits in vitro amyloid fibril formation.* Neurobiol Aging, 2004. **25**(8): p. 1033-43.
160. Tizon, B., et al., *Cystatin C protects neuronal cells from amyloid-beta-induced toxicity.* J Alzheimers Dis. **19**(3): p. 885-94.
161. Tizon, B., et al., *Induction of autophagy by cystatin C: a mechanism that protects murine primary cortical neurons and neuronal cell lines.* PLoS One. **5**(3): p. e9819.
162. Nairismagi, J., et al., *Progression of brain damage after status epilepticus and its association with epileptogenesis: a quantitative MRI study in a rat model of temporal lobe epilepsy.* Epilepsia, 2004. **45**(9): p. 1024-34.
163. Hasegawa, A., et al., *Regulation of glial development by cystatin C.* J Neurochem, 2007. **100**(1): p. 12-22.
164. Gravina, S.A., et al., *Amyloid beta protein (A beta) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at A beta 40 or A beta 42(43).* J Biol Chem, 1995. **270**(13): p. 7013-6.
165. Kawai, M., et al., *Degeneration of vascular muscle cells in cerebral amyloid angiopathy of Alzheimer disease.* Brain Res, 1993. **623**(1): p. 142-6.
166. Davis-Salinas, J., et al., *Thrombin receptor activation induces secretion and nonamyloidogenic processing of amyloid beta-protein precursor.* J Biol Chem, 1994. **269**(36): p. 22623-7.
167. Suo, Z., et al., *Soluble Alzheimers beta-amyloid constricts the cerebral vasculature in vivo.* Neurosci Lett, 1998. **257**(2): p. 77-80.
168. Dietrich, H.H., et al., *Soluble amyloid-beta, effect on cerebral arteriolar regulation and vascular cells.* Mol Neurodegener, 2010. **5**: p. 15.
169. McGowan, E., et al., *Abeta42 is essential for parenchymal and vascular amyloid deposition in mice.* Neuron, 2005. **47**(2): p. 191-9.
170. Han, B.H., et al., *Cerebrovascular dysfunction in amyloid precursor protein transgenic mice: contribution of soluble and insoluble amyloid-beta peptide, partial restoration via gamma-secretase inhibition.* J Neurosci, 2008. **28**(50): p. 13542-50.
171. Suo, Z., et al., *Superoxide free radical and intracellular calcium mediate A beta(1-42) induced endothelial toxicity.* Brain Res, 1997. **762**(1-2): p. 144-52.
172. Price, J.M., et al., *beta-Amyloid induces cerebrovascular endothelial dysfunction in the rat brain.* Neurol Res, 1997. **19**(5): p. 534-8.
173. Davis-Salinas, J. and W.E. Van Nostrand, *Amyloid beta-protein aggregation nullifies its pathologic properties in cultured cerebrovascular smooth muscle cells.* J Biol Chem, 1995. **270**(36): p. 20887-90.
174. Vilhjalmsson, D.T., H. Blondal, and F.R. Thormodsson, *Solubilized cystatin C amyloid is cytotoxic to cultured human cerebrovascular smooth muscle cells.* Exp Mol Pathol, 2007. **83**(3): p. 357-60.
175. Van Nostrand, W.E., J.P. Melchor, and L. Ruffini, *Pathologic amyloid beta-protein cell surface fibril assembly on cultured human cerebrovascular smooth muscle cells.* J Neurochem, 1998. **70**(1): p. 216-23.
176. Refolo, L.M., et al., *Nerve and epidermal growth factors induce the release of the Alzheimer amyloid precursor from PC 12 cell cultures.* Biochem Biophys Res Commun, 1989. **164**(2): p. 664-70.
177. Koh, J.Y., L.L. Yang, and C.W. Cotman, *Beta-amyloid protein increases the vulnerability of cultured cortical neurons to excitotoxic damage.* Brain Res, 1990. **533**(2): p. 315-20.
178. Wei, H., et al., *beta-amyloid peptide-induced death of PC 12 cells and cerebellar granule cell neurons is inhibited by long-term lithium treatment.* Eur J Pharmacol, 2000. **392**(3): p. 117-23.
179. Jayaprakasam, B., K. Padmanabhan, and M.G. Nair, *Withanamides in Withania somnifera fruit protect PC-12 cells from beta-amyloid responsible for Alzheimer's disease.* Phytother Res. **24**(6): p. 859-63.

180. Dewachter, I. and F. Van Leuven, *Secretases as targets for the treatment of Alzheimer's disease: the prospects.* Lancet Neurol, 2002. **1**(7): p. 409-16.
181. Pratico, D., *Evidence of oxidative stress in Alzheimer's disease brain and antioxidant therapy: lights and shadows.* Ann N Y Acad Sci, 2008. **1147**: p. 70-8.
182. Schindowski, K., K. Belarbi, and L. Buee, *Neurotrophic factors in Alzheimer's disease: role of axonal transport.* Genes Brain Behav, 2008. **7 Suppl 1**: p. 43-56.
183. Schenk, D., et al., *Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse.* Nature, 1999. **400**(6740): p. 173-7.
184. Levy, M.L., J.L. Cummings, and R. Kahn-Rose, *Neuropsychiatric symptoms and cholinergic therapy for Alzheimer's disease.* Gerontology, 1999. **45 Suppl 1**: p. 15-22.
185. Hashimoto, Y., et al., *A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Abeta.* Proc Natl Acad Sci U S A, 2001. **98**(11): p. 6336-41.
186. Tajima, H., et al., *Evidence for in vivo production of Humanin peptide, a neuroprotective factor against Alzheimer's disease-related insults.* Neurosci Lett, 2002. **324**(3): p. 227-31.
187. Hashimoto, Y., et al., *Detailed characterization of neuroprotection by a rescue factor humanin against various Alzheimer's disease-relevant insults.* J Neurosci, 2001. **21**(23): p. 9235-45.
188. Kariya, S., et al., *Humanin inhibits cell death of serum-deprived PC12h cells.* Neuroreport, 2002. **13**(6): p. 903-7.
189. Chiba, T., et al., *Development of a femtomolar-acting humanin derivative named colivelin by attaching activity-dependent neurotrophic factor to its N terminus: characterization of colivelin-mediated neuroprotection against Alzheimer's disease-relevant insults in vitro and in vivo.* J Neurosci, 2005. **25**(44): p. 10252-61.
190. Mamiya, T. and M. Ukai, *[Gly(14)]-Humanin improved the learning and memory impairment induced by scopolamine in vivo.* Br J Pharmacol, 2001. **134**(8): p. 1597-9.
191. Bachar, A.R., et al., *Humanin is expressed in human vascular walls and has a cytoprotective effect against oxidized LDL-induced oxidative stress.* Cardiovasc Res. **88**(2): p. 360-6.
192. Xu, X., et al., *Humanin is a novel neuroprotective agent against stroke.* Stroke, 2006. **37**(10): p. 2613-9.
193. Xu, X., et al., *Neuroprotective effect of humanin on cerebral ischemia/reperfusion injury is mediated by a PI3K/Akt pathway.* Brain Res, 2008. **1227**: p. 12-8.
194. Matsuoka, M., et al., *Humanin and colivelin: neuronal-death-suppressing peptides for Alzheimer's disease and amyotrophic lateral sclerosis.* CNS Drug Rev, 2006. **12**(2): p. 113-22.
195. Jung, S.S. and E. Levy, *Murine cerebrovascular cells as a cell culture model for cerebral amyloid angiopathy: isolation of smooth muscle and endothelial cells from mouse brain.* Methods Mol Biol, 2005. **299**: p. 211-9.
196. Van Nostrand, W.E., et al., *Cerebrovascular smooth muscle cell surface fibrillar A beta. Alteration of the proteolytic environment in the cerebral vessel wall.* Ann N Y Acad Sci, 2000. **903**: p. 89-96.
197. Melchor, J.P. and W.E. Van Nostrand, *Fibrillar amyloid beta-protein mediates the pathologic accumulation of its secreted precursor in human cerebrovascular smooth muscle cells.* J Biol Chem, 2000. **275**(13): p. 9782-91.
198. Jung, S.S. and W.E. Van Nostrand, *Humanin rescues human cerebrovascular smooth muscle cells from Abeta-induced toxicity.* J Neurochem, 2003. **84**(2): p. 266-72.
199. Meydani, M., *Vitamin E.* Lancet, 1995. **345**(8943): p. 170-5.
200. Vatassery, G.T., et al., *Effect of high doses of dietary vitamin E on the concentrations of vitamin E in several brain regions, plasma, liver, and adipose tissue of rats.* J Neurochem, 1988. **51**(2): p. 621-3.

201. Sano, M., et al., *A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study.* N Engl J Med, 1997. **336**(17): p. 1216-22.
202. Adams, J.D., Jr., et al., *Alzheimer's and Parkinson's disease. Brain levels of glutathione, glutathione disulfide, and vitamin E.* Mol Chem Neuropathol, 1991. **14**(3): p. 213-26.
203. Jackson, C.V., et al., *Vitamin E and Alzheimer's disease in subjects with Down's syndrome.* J Ment Defic Res, 1988. **32 (Pt 6)**: p. 479-84.
204. Sung, S., et al., *Early vitamin E supplementation in young but not aged mice reduces Abeta levels and amyloid deposition in a transgenic model of Alzheimer's disease.* FASEB J, 2004. **18**(2): p. 323-5.
205. Goodman, Y. and M.P. Mattson, *Secreted forms of beta-amyloid precursor protein protect hippocampal neurons against amyloid beta-peptide-induced oxidative injury.* Exp Neurol, 1994. **128**(1): p. 1-12.
206. Behl, C., et al., *Vitamin E protects nerve cells from amyloid beta protein toxicity.* Biochem Biophys Res Commun, 1992. **186**(2): p. 944-50.
207. Yatin, S.M., S. Varadarajan, and D.A. Butterfield, *Vitamin E Prevents Alzheimer's Amyloid beta-Peptide (1-42)-Induced Neuronal Protein Oxidation and Reactive Oxygen Species Production.* J Alzheimers Dis, 2000. **2**(2): p. 123-131.
208. Thomas, T., et al., *beta-Amyloid-mediated vasoactivity and vascular endothelial damage.* Nature, 1996. **380**(6570): p. 168-71.
209. Munoz, F.J., et al., *Vitamin E but not 17beta-estradiol protects against vascular toxicity induced by beta-amyloid wild type and the Dutch amyloid variant.* J Neurosci, 2002. **22**(8): p. 3081-9.
210. Gupta-Bansal, R., R.C. Frederickson, and K.R. Brunden, *Proteoglycan-mediated inhibition of A beta proteolysis. A potential cause of senile plaque accumulation.* J Biol Chem, 1995. **270**(31): p. 18666-71.
211. Gervais, F., et al., *Targeting soluble Abeta peptide with Tramiprosate for the treatment of brain amyloidosis.* Neurobiol Aging, 2007. **28**(4): p. 537-47.
212. Ghiso, J., et al., *Systemic catabolism of Alzheimer's Abeta40 and Abeta42.* J Biol Chem, 2004. **279**(44): p. 45897-908.
213. Santa-Maria, I., et al., *Tramiprosate, a drug of potential interest for the treatment of Alzheimer's disease, promotes an abnormal aggregation of tau.* Mol Neurodegener, 2007. **2**: p. 17.
214. Aisen, P.S., et al., *A Phase II study targeting amyloid-beta with 3APS in mild-to-moderate Alzheimer disease.* Neurology, 2006. **67**(10): p. 1757-63.
215. Aisen, P.S., et al., *Tramiprosate in mild-to-moderate Alzheimer's disease - a randomized, double-blind, placebo-controlled, multi-centre study (the Alphase Study).* Arch Med Sci. **7**(1): p. 102-11.
216. Greene, L.A., Sobeih, M. M., Teng, K. K., *Methodologies for the Culture and Experimental Use of the PC12 Rat Pheochromocytoma Cell Line.* Culturing Nerve Cells, ed. G. Banker, Goslin, K. 1991: Massachusetts Institude of Technology.
217. Vilhjalmsson, D.T., I.E. Ingolfsdottir, and F.R. Thormodsson, *Isolation of amyloid by solubilization in water.* Methods Mol Biol. **849**: p. 403-10.
218. Pras, M., et al., *The characterization of soluble amyloid prepared in water.* J Clin Invest, 1968. **47**(4): p. 924-33.
219. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.
220. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.
221. Switzer, R.C., 3rd, C.R. Merril, and S. Shifrin, *A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels.* Anal Biochem, 1979. **98**(1): p. 231-7.

222. Towbin, H., T. Staehelin, and J. Gordon, *Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979.* Biotechnology, 1992. **24**: p. 145-9.
223. Cree, I.A., *Luminescence-based cell viability testing.* Methods Mol Biol, 1998. **102**: p. 169-77.
224. Then, S.M., et al., *Is vitamin E toxic to neuron cells?* Cell Mol Neurobiol, 2009. **29**(4): p. 485-96.
225. Bitan, G. and D.B. Teplow, *Preparation of aggregate-free, low molecular weight amyloid-beta for assembly and toxicity assays.* Methods Mol Biol, 2005. **299**: p. 3-9.
226. Rangachari, V., et al., *Amyloid-beta(1-42) rapidly forms protofibrils and oligomers by distinct pathways in low concentrations of sodium dodecylsulfate.* Biochemistry, 2007. **46**(43): p. 12451-62.
227. Kaur, G. and E. Levy, *Cystatin C in Alzheimer's disease.* Front Mol Neurosci. **5**: p. 79.
228. Aho, L., et al., *Immunohistochemical visualization of amyloid-beta protein precursor and amyloid-beta in extra- and intracellular compartments in the human brain.* J Alzheimers Dis. **20**(4): p. 1015-28.